Title

Elevated microbial CO₂ production and fixation in the oxic/anoxic interface of estuarine water columns during seasonal anoxia

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Abstract

Gradients of dissolved oxygen concentrations in stratified estuarine water columns directly influence microbial composition and metabolic pathways, resulting in vertical and axial chemical gradients of redox-active species. Understanding such microbial responses to changing geochemical conditions and elucidating the diversity of microbially-mediated processes are needed to comprehensively identify ecosystem functions. We tested the hypothesis that microbial metabolic processes in the vicinity of the oxic/anoxic interface in Chesapeake Bay would be elevated due to the coincidence of oxidized and reduced compounds. We measured rates of microbial redox processes associated with carbon cycles and quantified geochemical constituents. The transition from oxic to anoxic to oxic conditions was correlated with the pattern of dissolved nitrogen species, with the most reducing conditions comprising the highest concentrations of dissolved chemical compounds. The vertical distribution of community respiration was measured with changes in dissolved inorganic carbon concentrations and was related to water column stratification, with the highest median and variability ($1.2 \pm 1.4 \mu\text{mol L}^{-1} \text{h}^{-1}$) in the most stratified conditions. Rates of CO_2 fixation under dark conditions, a measure of chemoautotrophy, were elevated at the base of oxyclines and significantly correlated with a gradient of density and some reduced compounds. Although vertical interpolation must be made with caution due to high vertical variability, chemoautotrophic production averaged $0.5 \text{ mmol m}^{-2} \text{h}^{-1}$ from May to August and added 5.8 % to autochthonous organic carbon production in the mesohaline Chesapeake Bay. Overall, our results suggest that anaerobic community metabolism and chemoautotrophy in the oxic/anoxic interface exert a small impact on estuarine carbon cycles.

1. Introduction

Estuarine ecosystems contain a series of unique and mixed microbial communities that originate within the estuary and from neighboring ecosystems, such as rivers, oceans, and watershed soils (Crump et al. 2004). These microbes acclimate to stable or widely ranging environmental conditions (Bouvier and Del Giorgio 2002; Fortunato and Crump 2011) and many are capable of fulfilling thermodynamic energy requirements in an annually recurring environmental pattern (Bouskill et al. 2011). For example, if dissolved oxygen (DO) is depleted due to a combination of biochemical (e.g., organic matter oxidation) and physical (e.g., stratification) mechanisms during seasonal anoxic events, microbes use alternative electron acceptors for respiration, such as NO_3^- , Mn(IV), Fe(III), and SO_4^{2-} (Froelich et al. 1979; Wright et al. 2012). The dynamic response of microbial processes is demonstrated by metatranscriptomic analyses, revealing that successional transitions of respiratory processes corresponded to a given geochemical condition in anoxic waters (Eggleston et al. 2014; Hewson et al. 2014).

The adaptability of the microbial community to changing redox conditions allows microbial specialists to thrive in the vicinity of the oxic/anoxic interface (Brune et al. 2000). One of best-studied examples of microbial diversity are active chemoautotrophic and anoxygenic photoautotrophic processes attributed to the simultaneous presence of various oxidized and reduced compounds. The vertical distribution of the microbes is strongly related to the vertical structure of oxic/anoxic or oxic/sulfidic interfaces (Sorokin et al. 1995; Casamayor 2010; Casamayor et al. 2012). In some ecosystems where photosynthetic production is restricted to upper mixed layers, chemoautotrophic production can support a deep secondary microbial food web (Ho et al. 2004; Alonso-Sáez et al. 2010; Swan et al. 2011). Surprisingly, zooplankton have

been shown to fulfill about half of their total carbon requirements through the consumption of chemoautotrophic and anoxygenic photoautotrophic production in a shallow ecosystem (Camacho et al. 2001). Chemoautotrophy and anoxygenic photoautotrophy have been investigated in stratified ecosystems, including lagoons (Llirós et al. 2011), lakes (Cloern et al. 1983), fjords (Zopfi et al. 2001), estuaries (Casamayor et al. 2001), and oceans (Taylor et al. 2001; Labrenz et al. 2005). However, few such studies have been conducted in eutrophic estuaries due to an emphasis on phytoplankton production dominating estuarine food webs and carbon cycling.

Previous studies have suggested spatiotemporal variability in microbial phylogenetic and functional composition reflects the diversity of metabolic processes in the Chesapeake Bay during seasonal anoxic events (Crump et al. 2007; Eggleston et al. 2014; Hewson et al. 2014; Lee et al. 2015). For example, occasional high production of dissolved inorganic carbon ($\text{DIC} = [\text{H}_2\text{CO}_3] + [\text{HCO}_3^-] + [\text{CO}_3^{2-}]$) near the top of oxyclines and net consumption of DIC in the oxic/anoxic interface suggested not only close proximity between aerobic heterotrophs and chemoautotrophs, but also the formation of a secondary microbial food web. Net consumption of DIC was shown to intensify following experimental augmentation of samples with terminal electron acceptors (e.g., O_2 , NO_3^-), suggesting enhanced aerobic and anaerobic chemoautotrophy (Lee et al. 2015). In addition, vertical mixing from frequent and strong winds during autumn was coincident with increases in *Thaumarchaeote* transcripts and NO_2^- concentration in the dissolved inorganic nitrogen ($\text{DIN} = [\text{NH}_4^+] + [\text{NO}_2^-] + [\text{NO}_3^-]$) pool. The decrease in denitrification related transcript ratios relative to spring and summer suggested that nitrification rather than denitrification was responsible for NO_2^- accumulation (Eggleston et al. 2014). Further, assuming no limitation of electron acceptors and donors under optimal growth conditions for

chemoautotrophy, the upward flux of reduced nitrogen and sulfur compounds across oxyclines can potentially fix up to $9.7 \text{ mmol C m}^{-2} \text{ day}^{-1}$ in summer (Lee et al. 2015). This study concluded that chemoautotrophy would exert a small impact on estuarine organic and inorganic carbon cycles due to the higher magnitude of phytoplankton production and aerobic respiration. However, it is still important to understand chemoautotrophy because it couples the fixation of CO_2 with oxidation of reduced compounds that can be further recycled for other electron accepting processes. In addition, the strong association of chemoautotrophy with stratification could cause large variability in the extent of chemoautotrophic metabolism because large interannual variability in river discharge, controlling the extent of stratification (Hagy et al. 2004), affects the fluxes of electron acceptors and donors.

The goal of the present study was to investigate the spatiotemporal variability of community metabolism across DO and redox gradients to assess the contribution of different biological redox processes on estuarine carbon cycles. Further, we identified and quantified the association of community metabolism with a gradient of environmental and geochemical factors to explain additional variability in various metabolic processes in the oxic/anoxic interface. We hypothesize that anoxygenic photoautotrophic production (AnoxyPP) and primary production in dark (DarkPP) would be elevated during seasonal anoxic events due to the close proximity between the abundant supply of oxidized and reduced compounds in the interface.

2. Materials and methods

Pelagic and benthic samples were collected during seven temporal surveys at S3 (38°33'54" N, 76°26'32" W) located in the mesohaline region of Chesapeake Bay from spring to fall (18 April, 24 May, 14 June, 8 July, 8 August, 30 August, and 21 September 2011) (Fig. 1). We also conducted spatial surveys at five stations (S1 to S5) along the 150-km long main axis from near the mouth of Potomac River to the Chesapeake Bay Bridge on 6-12 July 2011 on board the R/V Hugh R. Sharp.

We measured conductivity, temperature, and depth (CTD) using a profiler (SBE25, SBE 911plus, Sea-Bird Electronics) equipped with DO (SBE 43, Sea-Bird Electronics) and photosynthetically active radiation (PAR) sensors (400-700 nm, Biospherical Instruments). On 6 and 12 July during the transect survey, water column geochemistry was surveyed with an undulating towed vehicle (Scanfish II-1250, GMI Denmark) equipped with CTD (SBE25), DO (SBE 43), and fluorometer (WET Labs) sensors. The surveys began from S1 at the Potomac River mouth to S5 at the Chesapeake Bay Bridge for ~12 hours (h).

We collected samples using a diaphragm pump with a 2.5-cm diameter hose attached to the CTD frame and terminated shipboard with a conical PVC manifold water distribution system which allowed multiple sample vials to be filled simultaneously. Samples were collected at eight depths (three above, two within, and three below oxyclines) between 08:30 h and 11:30 h; when an oxycline was absent salinities were used to apportion sampling depths.

Solute samples were filtered through 0.45 μm cellulose acetate syringe filters and samples collected for NO_2^- , NO_3^- , NH_4^+ , total dissolved sulfide ($\text{DS} = [\text{S}^{2-}] + [\text{HS}^-] + [\text{H}_2\text{S}]$), soluble reactive phosphorus (SRP), dissolved manganese ($\text{DMn} = [\text{Mn(II)}] + [\text{Mn(III)}]$), and

dissolved iron (DFe = Fe(II)+ Fe(III)) in 7 ml polyethylene vials with screw caps. Concentrations of NH_4^+ , DS, SRP, DMn, and DFe were determined colorimetrically (Brewer and Spencer 1971; Parsons et al. 1984) with a spectrophotometer (UVmini-1240, Shimadzu), while NO_2^- and NO_3^- were determined using segmented flow analysis after cadmium reduction by the Horn Point Analytical Services (Lane et al. 2000). Samples for fluorometric pigment analysis (50 ml) were collected in duplicate, filtered through 25 mm GF/F filters and stored at -20 °C. The concentration of chlorophyll a and pheophytin was determined using EPA method 445.0 (Arar and Collins 1997).

Both DO and DIC-based respiration measurements were made in triplicate dark 60 ml biochemical oxygen demand (BOD) bottles. Samples for initial concentrations were preserved with 10 μl of 50 % saturated mercuric chloride in triplicate 12 ml glass vials with butyl rubber septum caps, while the BOD bottles for final concentrations were covered with opaque plastic bags and incubated at in situ water temperatures ($\pm 1^\circ\text{C}$) for 24 h. At the end of incubation samples were siphoned into the vials and preserved with Hg. Capped vials were submerged in an opaque container below incubation temperatures and analyzed within three days. Respiration rates were computed as concentration differences between initial and final samples divided by incubation time. A linear increase (or decrease) of DIC (DO) in similar samples was observed during previous incubations up to 40 h (Lee et al. 2015).

A DIC analyzer, consisting of an automated digital pump, a mass-flow-controller, and an infrared CO_2 detector (LI-COR), was used (Apollo SciTech; Cai and Wang 1998). Certified reference material (provided by A. Dickson at Scripps Institution of Oceanography) was used to calibrate the analyzer according to Dickson et al. (2007) prior to analyzing the first sample of

each cruise and daily during the transect survey. Overall, the calibration coefficient of variation ranged from 0.05 to 0.12 % with an average standard deviation of $\pm 1.5 \mu\text{mol L}^{-1}$.

Dissolved oxygen to argon ratios were determined with a membrane inlet mass spectrometer (Kana et al. 1994) yielding a precision of <0.05 %. Thermostated (± 0.02 °C of incubation temperature) deionized water was used for standardization and gas ratios were converted to gas concentrations using temperature and salinity-based argon concentrations (Colt 1984).

Cellular CO₂ fixation was estimated using radiolabeled NaH¹⁴CO₃ (¹⁴CO₂, PerkinElmer) as described in previous studies (Kemp et al. 1993; García-Cantizano et al. 2005). A working ¹⁴CO₂ stock was prepared using a sodium bicarbonate solution before the dilution of ¹⁴CO₂ to a final concentration of 18.5 Bq (5 μCi) per sample in an anoxic glove bag. Water samples from the third to sixth depths were collected in triplicate 12 ml vials with butyl rubber septum caps for determining photosynthetic fixation, including oxygenic photoautotrophic production (OxyPP) and AnoxyPP. Similarly, water samples were collected from the third to eighth depths for determining DarkPP. The ¹⁴CO₂ stock was added into each vial through the septa with a gas-tight Hamilton syringe in an anoxic glove bag. Four treatments were prepared and incubated for 4 h in the bag in a temperature-controlled environmental chamber. We checked the validity of consistent ¹⁴CO₂ fixation over the course of incubation and the result is provided in Supplementary material Figure S1.

The first set of samples was covered with neutral-density screens allowing passage of four PAR levels (6.1, 3.7, 2.2, and 1.3 % of surface irradiance (E_0)). The second set of triplicate samples was incubated under the same PAR with an addition of 3-(3', 4'-dichlorophenyl)-1,1'-

dimethyl urea (DCMU) dissolved in ethanol at a final concentration of $2 \mu\text{mol L}^{-1}$ to inhibit the photosystem II reaction. The third set of samples was incubated in the dark. The last set of samples was incubated with formaldehyde (4 %, volume:volume) to correct for abiotic reactions. At the end of incubation, samples were immediately filtered onto $0.2 \mu\text{m}$ polycarbonate membrane filters (Millipore) and dried 24 h. Then, filters were fumed with hydrogen chloride for 30 min and immersed in scintillation cocktail (PerkinElmer).

Radioactivity was counted in a liquid scintillation analyzer (Packard Tri-Carb 3100TR) with quench correction. The $^{14}\text{CO}_2$ assimilation by OxyPP was calculated by subtracting the $^{14}\text{CO}_2$ activity in illuminated vials with DCMU from that in the illuminated vials. The $^{14}\text{CO}_2$ assimilation by Anoxygenic PP (AnoxyPP) was calculated by subtracting the activity in dark vials from that in illuminated vials with DCMU. The $^{14}\text{CO}_2$ assimilation by Dark PP (DarkPP) was calculated by subtracting the $^{14}\text{CO}_2$ activity in killed samples from that in dark vials.

Bacterial production (BP) was estimated to correct for heterotrophic anaplerotic carboxylation, likely overestimating chemoautotrophy. Water samples for BP measurements were pumped directly from below pycnoclines to the bottom of 120 ml BOD bottles and allowed to overflow three times to ensure no bubbles remained. Samples were sealed and stored under water in the dark at ambient bottom water temperature during transport and were processed immediately upon return from the station (<5 h). Heterotrophic BP was measured by determining the rate of ^3H -leucine incorporation during 1-h incubations. All incubations were conducted in an anaerobic chamber (Coy) in sealed plastic syringes with the headspace removed. All plastic was stored in an O_2 -free environment 24 h prior to use. Carbon production was calculated based on leucine incorporation using a ratio of cellular carbon to protein of 0.86, a fraction of leucine in

protein of 0.073, and an intracellular leucine isotope dilution of two (Kirchman 1993; Crump et al. 2007).

Sediment cores were collected using a box corer to determine sediment-water flux at S3 during the temporal survey. The sediments were sub-cored using three 7×30 cm (inner diameter × height) acrylic cylinders to a depth of ~15 cm. The sediment cores plus a core filled only with bottom water were incubated in the dark with stirring, and four samples were collected over 6 or 18-h time courses for aerobic or anaerobic incubations, respectively. For DIC analysis samples were collected in glass vials with preservation using Hg. Samples for NH_4^+ and NO_x ($\text{NO}_2^- + \text{NO}_3^-$) analysis were collected in syringes and filtered prior to preservation via freezing. Overall, linear regression of overlying water DIC and DIN time courses resulted in high regression coefficients ($n = 21$, $R^2 > 0.90$, $p < 0.05$).

All statistical tests were performed using R (R Foundation). Correlation and multiple regression analysis were used to identify the relationship between microbial metabolism and a vertical slope (gradient) of diverse variables. Gradients were calculated by subtracting values of shallower from deeper depth between neighboring sampling depths, and the difference was normalized by depth difference (i.e., $= (C_{i+1} - C_i) \times (Z_{i+1} - Z_i)^{-1}$, where C and Z indicates value and depth, respectively). For multiple regression analysis, absolute values of gradients were used to examine the effect of the magnitude of gradients, rather than both magnitude and direction (i.e., sign). In model selection, we used two criteria to determine best models: the Bayesian information criterion (BIC) and Mallows' Cp (Cp), satisfying both >10 BIC (Kass and Raftery 1995) and <1 difference between Cp values and the number of variables used for each model plus one (Gilmour 1996), respectively. Then, we present one model with the highest regression coefficient from a set of models with the same number of variables. Lastly, geochemical water

properties were mapped with contour plots using EasyKrigV3.0 in MATLAB (Mathworks). We used kriging with an isotropic nonlinear semi-variogram model as a gridding method that performed statistical interpolation at every 5 cm in the y-direction, and 12 h and 1 km in the x-direction for temporal and transect survey, respectively.

3. Results

3.1. Environmental conditions

Seasonal and spatial changes in density gradient reflected the effect of high river discharge with the initial development and persistent formation of pycnoclines in late spring (Fig. 2a) and throughout the mesohaline region in summer (Fig. 2c). In addition, the depth of maximum buoyancy frequency (i.e., natural logarithm of N^2), an index of vertical stability, was observed in the pycnocline and coincided with observations of the oxyclines (Fig. 2b, d). The seasonal anoxic event was terminated by Hurricane Irene (28 August), resulting in earlier destratification and reoxygenation of water columns for about a month. In addition, the average thickness of oxyclines, determined using a threshold value of conductivity gradient defined by the Chesapeake Bay Program (<http://www.chesapeakebay.net/data>), was 3.0 m in spring and summer 2011, which is shallower than the 4.5 m observed in 2010 (Lee et al. 2015).

3.2. Temporal survey

Observations of the transition of DO below oxyclines showed a transition through oxic, hypoxic, anoxic (i.e., no DO and DS), sulfidic, and back to oxic condition over about four months (Fig. 3a). The distribution of fluorometric pigments suggested that the seasonal oxic transition was initiated by microbial DO consumption of algal-derived organic matter in spring (Fig. 3c, d). The same biophysical mechanisms (i.e., respiration, oxycline) were also reflected in the pattern of DIC that accumulated below the oxycline (Fig. 3h). Overall, the transition of DIN best reflected that of redox conditions likely due to various microbial-mediated nitrogen processes (Fig. 3e, f, g). Oxidized forms of DIN were supplied by rivers in spring and coincided with a spring bloom of phytoplankton, suggesting a rapid consumption of DIN and production of

organic nitrogen. Relative increase of lower oxidation states of DIN (i.e., NH_4^+) towards deeper depths and warmer months was likely caused by microbial consumption of organic nitrogen. Finally, NO_2^- and NO_3^- concentrations rebounded after Hurricane Irene and were even higher than the concentration in spring, an observation not previously recorded.

Despite large interannual differences in environmental conditions, the seasonal pattern and magnitude of respiration in 2011 (Fig. 4) was similar to those in 2010 (Lee et al. 2015). We observed high vertical variability and maximum respiration rates near the top of the oxycline in summer (Fig. 4c, d). On 14 June, the concentrations of NO_2^- ($3.3 \mu\text{mol L}^{-1}$), DS ($1.2 \mu\text{mol L}^{-1}$), DMn ($1.5 \mu\text{mol L}^{-1}$), and DFe ($1.2 \mu\text{mol L}^{-1}$) approached zero at the depth of the observed subsurface respiration maximum. On 8 July when the oxycline was the shallowest among the surveys (e.g., 6.9 m for $0.5 \text{ mg O}_2 \text{ L}^{-1}$ isopleth), we also observed the coincidence of maximum respiration and the steepest gradient of reduced species.

DarkPP showed strong vertical gradients when the water column was stratified, with high rates consistently observed below the depth of the $0.5 \text{ mg O}_2 \text{ L}^{-1}$ isopleth in summer (Fig. 4). The depth-integrated DarkPP rates below the oxycline were 39.7, 17.9, 26.8, and $9.7 \text{ mmol m}^{-2} \text{ day}^{-1}$ on 24 May, 14 June, 8 July, and 8 August, respectively. To convert volumetric hourly rates to areal daily rates, we assumed a consistent thickness and formation of oxyclines over tidal cycles (24 h), supported by the result of a continuous density survey using an undulating towed vehicle in July (data not shown). It should be noted that average $4.4 \pm 1.4 \text{ mmol m}^{-2} \text{ day}^{-1}$ of $^{14}\text{CO}_2$ fixation was also detected in non-stratified/oxic water columns (Fig. 4a, f, g), suggesting that non-chemoautotrophic $^{14}\text{CO}_2$ fixation consistently occurred regardless of oxic conditions.

Additionally, a comparison between OxyPP and AnoxyPP during the temporal survey is shown in different irradiance levels (Supplementary material Figure S2). High OxyPP rates were observed at 6.1 and 3.7 % E_0 , while similar and even higher AnoxyPP were measured at lower irradiance when sulfidic conditions ($>11 \mu\text{mol L}^{-1}$) prevailed below the oxycline. Additionally, to check the effect of ethanol in DCMU on $^{14}\text{CO}_2$ fixation, we incubated additional sets of samples augmented with the same amount of ethanol in DCMU. Although there was no statistical difference in the change of $^{14}\text{CO}_2$ fixation over time between samples with and without ethanol (analysis of covariance: $p > 0.2$, graph not shown), we sometimes observed up to 7 % of elevated fixation in ethanol samples collected near the top of oxyclines.

The molar DIC:DIN ratio of sediment fluxes averaged 7.4 ± 0.4 , similar to the Redfield ratio of 6.6. The sediment flux of DIC ranged from 35.2 to $56.3 \text{ mmol m}^{-2} \text{ day}^{-1}$ with lower fluxes in summer than spring and fall, while that of DIN (i.e., the sum of NH_4^+ and NO_x fluxes) ranged from 3.6 to $7.5 \text{ mmol m}^{-2} \text{ day}^{-1}$ with the same seasonal pattern (Supplementary material Figure S3).

3.3. Spatial transect survey

The pattern of geochemical constituents during the transect survey showed the origin of reduced compounds (between S4 and S5) where anoxia is first observed each year (Fig. 5). The 2011 record-high anoxic event resulted in a full coverage of anoxic waters from the northern to southern limits of the mesohaline region. The concentration of reduced compounds (e.g., NH_4^+ , DS, SRP, DMn) in bottom waters increased northward as the bottom water aged. However, the transformation of NO_x was not observed as in the temporal survey.

Consistent with the results of the temporal survey, higher subsurface respiration rates were observed when high concentrations of reduced compounds prevailed below oxyclines (Fig. 6c, d, e). In addition, high DarkPP near the base of the oxycline was a consistent feature. Depth-integrated DarkPP below the oxycline were 22.2, 30.3, 26.8, 60.4, and 24.6 mmol m⁻² day⁻¹ from S1 to S5, respectively. The highest DarkPP of 0.43 μmol L⁻¹ h⁻¹ was measured at S4 and S5 where water depths decrease sharply to the north (Fig. 6d, e).

3.4. Statistical analysis

The results of correlation analyses using values (i.e., concentrations) and gradients were notably different for some variables (Table 1). For example, temperature correlated with pheophytin, NO₃⁻ and NH₄⁺, but the gradient of temperature correlated with gradients of density, DO, NH₄⁺, DS, and DMn. Also, NO₃⁻ concentration appeared to be a good predictor for several geochemical properties, but the gradient of NO₃⁻ was not correlated with any variable.

Multiple regression analysis was also conducted using gradients of variables to observe relationships of microbial metabolism with degrees of changes in environmental and geochemical conditions. Note that all gradients were converted to absolute values (i.e., no sign) to only investigate the effect of magnitude and to simplify the interpretation of results. The models selected according to the predetermined criteria (i.e., BIC, C_p) showed different responses of respiration over oxic conditions (Supplementary material Table S1). The gradient in aerobic respiration was positively associated with gradients of temperature and chlorophyll a, while it was negatively associated with gradients of NO₃⁻ and DMn. Contrary, the gradient of respiration in hypoxic and anoxic condition was only significantly associated with gradients of DFe and DMn. Thus, the results suggest a large discrepancy in the physiological responses of

aerobic and anaerobic organisms. On the other hand, the results of multiple regression analysis for gradients of DarkPP suggested that a gradient of geochemical variables can be used to predict DarkPP (Supplementary material Table S2); gradients of density, DO, DS, DFe, and DMn particularly exerted a strong influence ($p < 0.05$) on DarkPP variability, suggesting that opposing gradient systems in the diffusion-limited region supported DarkPP.

4. Discussion

Elevated community metabolism in the oxic/anoxic interface and the association with gradients of redox-related variables suggest that during seasonal anoxic events hydrodynamic and geochemical characteristics of the interface support microbial food webs near oxyclines. Average Susquehanna River discharge in March ($4,417 \text{ m}^3 \text{ s}^{-1}$) was the highest recorded since monitoring began in 1968 (<http://waterdata.usgs.gov/md/nwis>). In addition, the depth of the $2 \text{ mg O}_2 \text{ L}^{-1}$ isopleth was 2-4 m shallower in summer 2011 than 2010 (Lee et al. 2015). As a result, hypoxic ($<2 \text{ mg L}^{-1}$, $<62.5 \mu\text{mol L}^{-1}$) waters occupied 33 % (7.1 km^3) of the Maryland portion of the mainstem Chesapeake Bay in summer, which is greater than the long-term average of 4.8 km^3 from 1985 to 2010 (<http://mddnr.chesapeakebay.net/eyesonthebay>).

Despite interannual differences in the extent of stratification and hypoxic volume, we observed similar patterns of geochemical constituents compared to previous studies (Gavis and Grant 1986; Lee et al. 2015), suggesting conserved residents or recurring populations in bacterioplankton communities. The vertical patterns of respiration and autotrophic dark $^{14}\text{CO}_2$ fixation, elevated near the maximum stratification, also suggest that some subset of the microbial community was optimally adapted to the interface. Dynamic interactions between environmental conditions and microbial responses that drive productivity at these interfaces are discussed below, along with some implications for estuarine carbon cycles.

4.1. Metabolic response to stratification related variables

In the studied region, river discharge is positively correlated with phytoplankton biomass (Harding 1994; Adolf et al. 2006) and stratification (Hagy et al. 2004), while it is not correlated with phytoplankton sedimentation (Hagy et al. 2005). In general, high river discharges in 2011

resulted in higher community respiration than average anoxic years: a 1.5-fold increase of depth-integrated respiration in summer 2011 ($316.3 \text{ mmol m}^{-2} \text{ day}^{-1}$) than 2010 ($206.5 \text{ mmol m}^{-2} \text{ day}^{-1}$; Lee et al. 2015). However, sediment-water DIC fluxes were lower in summer 2011 (Supplementary material Figure S3) than 2010, suggesting a relative increase in pelagic metabolism and decrease in benthic metabolism during severe anoxic events.

The influence of stratification on geochemical variables and community metabolism has not been systematically assessed before, although most environmental and geochemical factors are affected by density to some extent in a stratified estuary. Thus, we investigated the relationship using both a value of geochemical variables at depths and a gradient of variables across neighboring sampling depths; the latter appears to be more appropriate for stratified ecosystems because density and chemical gradients determine diffusive and advective fluxes. Considering the importance of temperature on the variability in respiration in summer (Shiah and Ducklow 1994), the elevated respiration in the oxic/anoxic interface is counterintuitive due to a significant inverse relationship between a gradient of density and temperature (Table 1). The abrupt decrease in temperature could give the microbes in the interface a physiological disadvantage. However, the disadvantage may not have affected community respiration in the interface if a mixed microbial community occupied the region because of the inverse effect of temperature on aerobic and anaerobic respiration (Supplementary material Table S1). In addition, it is commonly found that the coexistence of aerobic and anaerobic communities in various environments benefits from syntrophic interactions (Gottschal and Szewzyk 1985; van den Ende et al. 1997). Thus, the coexistence of microbes with different metabolic strategies appears to be a crucial factor resulting in high respiration in the interface. Furthermore, over the entire study, stratification-dependent respiration profiles showed the highest variability and highest median

rate in the most stratified water columns although they were not significantly different (ANOVA, $p > 0.05$) (Supplementary material Figure S4). Respiration normalized to chlorophyll a concentration also yielded the same pattern as un-normalized respiration, suggesting relative increases in quantity or quality of organic matter near the maximum stratification.

4.2. Anoxygenic photoautotrophy

The results of AnoxyPP support the idea that the oxic/anoxic interface provided an optimal condition for a guild of photoautotrophic organisms (Supplementary material Figure S2). On 14 June, high AnoxyPP was measured at 2.2 % E_0 and coincided with $3.3 \mu\text{mol L}^{-1}$ of NO_2^- , suggesting NO_2^- might serve as an electron donor for purple sulfur bacteria (Griffin et al. 2007). On 8 July, AnoxyPP at 2.2 and 1.3 % E_0 coincided with DS removal rates of 1.5 and $1.8 \mu\text{mol L}^{-1} \text{h}^{-1}$, respectively. This observation suggests that 6.0 and $15.1 \mu\text{mol L}^{-1}$ DS was oxidized to fix $1 \mu\text{mol L}^{-1} \text{CO}_2$ (i.e., yield factor) at 2.2 and 1.3 % E_0 , respectively. The range of yield factors is in agreement with a previous study (Zopfi et al. 2001) and reflects the highly variable nature of AnoxyPP within the oxycline. While the majority of photosynthesis was contributed by OxyPP, our results suggest that the vertical shift of oxic conditions and the concurrent change of water column geochemistry may increase the patchy distribution of AnoxyPP.

However, the interpretation of AnoxyPP results must be made with caution because of likely contamination of cyanobacterial AnoxyPP and a positive feedback effect of heterotrophic nutrient recycling for AnoxyPP above the oxycline in summer. Cyanobacteria are capable of switching from oxygenic to anoxygenic metabolism in reducing environments (Jørgensen 1982). Among major taxonomic groups of phytoplankton in the Chesapeake Bay, a rapid increase of cyanobacteria from 2 to 26 % of total algal pigments from spring to summer (Adolf et al. 2006)

increases the possibility of cyanobacterial AnoxyPP. Thus, some of our samples with high chlorophyll a were possibly affected by cyanobacterial AnoxyPP in summer: the concentration was $>10.0 \mu\text{g L}^{-1}$ at 6.1 and 3.7 % E_0 , while it was $<2 \mu\text{g L}^{-1}$ at 2.2 and 1.3 % E_0 . However, it is impossible to quantitatively discriminate the contribution of cyanobacteria on total AnoxyPP for current approaches. Due to this methodological issue and possible overestimation of light-dependent $^{14}\text{CO}_2$ fixation, AnoxyPP was not incorporated into the carbon budget.

4.3. Chemoautotrophy

Similar to the results of respiration and AnoxyPP, stratification appeared to provide an optimal condition for chemoautotrophic growth because of consistently measured high rates near oxyclines (Fig. 4, Fig. 6). Despite the apparent vertical and seasonal pattern, most previous studies found weak or no correlation between chemoautotrophy and geochemical variables presumably due to the coexistence of a diverse assemblage of physiological and ecological types of chemoautotrophs (Casamayor 2010; Casamayor et al. 2012). However, we found significant models with a gradient of some geochemical variables, including density, DO, DS, DFe, and DMn for predicting DarkPP variability (Supplementary material Table S2); however, it remains difficult to determine the physiological requirement of DarkPP from the models because the relationship could be coincidental with geochemical changes.

We should note that our DarkPP results likely include ubiquitous anaplerotic carboxylation (Li 1982). Heterotrophs can also incorporate CO_2 using carboxylation reactions. In particular, heterotrophic CO_2 fixation can be enhanced under nutrient-limited conditions (Perez and Martin 1982; Alonso-Sáez et al. 2010), which is uncommon for bacterioplankton in the Chesapeake Bay (Jonas 1997). If we assume that measured DarkPP in non-stratified/oxic waters

only resulted from the carboxylation reaction, the reaction accounted for 6.4 to 11.7 % of BP in April, August, and September. Previous studies reported a similar range (1.4 to 13 %) depending on physiological and environmental conditions (Perez and Matin 1982; Roslev et al. 2004). Thus, heterotrophic carboxylation was assumed to have occurred throughout this study and all DarkPP results were corrected for averaged carboxylation reaction.

4.4. Implication of chemoautotrophy for chemical cycles

Chemoautotrophy is a generally inefficient and slow process due to high energy requirement for CO₂ fixation (Shively et al. 1998). Despite slow growth, chemoautotrophy can exert considerable influence on the dynamics of chemical cycles because the low energy yield of chemoautotrophy requires an oxidation of disproportionally large amounts of reduced substrates to fix CO₂. For example, the oxidation of 2 to 60 $\mu\text{mol L}^{-1}$ of NH₄⁺ and 1.7 to 9.1 $\mu\text{mol L}^{-1}$ of DS is required by chemoautotrophs to fix 1 $\mu\text{mol L}^{-1}$ CO₂ depending on physiological and environmental conditions controlling growth efficiency (Zopfi et al. 2001; Andersson et al. 2006; Veuger et al. 2013). Assuming only nitrification occurred on 24 May (no DS measured below oxyclines), average DarkPP of 0.1 $\mu\text{mol L}^{-1} \text{ h}^{-1}$ coincided with an average NH₄⁺ oxidation of 3.7 $\mu\text{mol L}^{-1} \text{ h}^{-1}$, measured simultaneously using a pair of identical water samples. Similarly, average DarkPP of 0.3 $\mu\text{mol L}^{-1} \text{ h}^{-1}$ on 8 July coincided with an average DS oxidation of 1.6 $\mu\text{mol L}^{-1} \text{ h}^{-1}$. Since our assumption of uniform chemoautotrophy (i.e., either NH₄⁺ or DS oxidization) is highly unlikely, it is probable that a guild of chemoautotrophs would simultaneously mediate a conversion of a series of different compounds aerobically and anaerobically (Kepkay and Nealson 1987; Straub et al. 1996; Benz et al. 1998).

4.5. Electron budget in the oxic/anoxic interface

The quantitative significance of different electron accepting processes was evaluated using the vertical profiles of oxidants and electron donors in the oxic/anoxic interface. This analysis helps us to estimate the balance of various opposing gradients and to assess potential metabolic processes. Vertical gradient of chemical concentrations is a key component of vertical dispersion of chemical species during seasonal anoxia as well as vertical advection (Hagy 2002). We estimated a vertical dispersion coefficient using a relationship between the flux of chemical species across the sediment-water interfaces and oxyclines:

$$\text{Sediment-water } \text{NH}_4^+ \text{ flux} = K ((\Delta\text{NH}_4^+) \times (\Delta\text{Depth})^{-1})$$

where K is the vertical dispersion coefficient and $((\Delta\text{NH}_4^+) \times (\Delta\text{Depth})^{-1})$ represents a gradient of NH_4^+ across oxyclines. We used NH_4^+ fluxes and concentrations because they were consistently measured throughout this study and had slow biogeochemical turnover rates. Although the equation does not explicitly include a vertical advection term, a gradient of chemical compounds is equally dependent of both diffusion and advection (Kemp et al. 1992). As a result, the estimated average K value of $0.13 \text{ cm}^2 \text{ s}^{-1}$, which is in agreement with previous studies (Kemp et al. 1992; Hagy 2002), from May to August was used for different chemical species (e.g., DO, NO_3^- , NO_2^- , DS, NH_4^+ , Mn, Fe) in each cruise.

The flux of electron acceptors and donors across oxyclines (computed by a gradient of chemical species multiplied by K) was converted to electron equivalents to evaluate a redox balance (Table 2). The downward flux of electron acceptors usually exceeded the upward flux of electron donors, indicated by net negative balance in Table 2, mainly due to excess DO flux. The contribution of electron acceptors other than DO to total electron balance was 5.6 % with the second highest contribution from NO_x (3.5 %). The upward flux of DMn and DFe was assumed

to be equal to the downward flux of Mn(IV) and Fe(III)-oxides due to the absence of vertical pattern and a rapid biochemical recycling of the two elements (Nealson and Myers 1992).

To estimate chemoautotrophic rates from the pattern of electron donors, we assumed that there was no limitation of electron acceptors as Table 2 suggested. We also assume maximum efficiency of yield factors (i.e., least amount of electron donor is oxidized to fix 1 unit of CO₂) for a series of chemoautotrophy, such as 0.58, 0.11, 0.32 for the oxidation of DS, NH₄⁺, and metal (Mn, Fe) oxides, respectively (Kepkay and Nealson 1987; Zopfi et al. 2001). As a result, the average depth-integrated potential chemoautotrophic rate across the oxycline was 5.4 mmol m⁻² day⁻¹ in summer, which accounts for 40 % of depth-integrated DarkPP throughout bottom mixed layers in summer (14.0 mmol m⁻² day⁻¹), suggesting the disproportionally large contribution of chemoautotrophy in the oxycline.

4.6. Carbon budget during seasonal anoxic event

The most obvious feature in the carbon budget is the dominance of phytoplankton production and pelagic aerobic respiration for the gain and loss of organic carbon, respectively (Fig. 7). We used gross phytoplankton production measured using DO balances (Smith and Kemp 2001) and converted to carbon equivalent using a photosynthetic quotient of 1.04 (Lee et al. 2015). The production was measured in 1996 and 1997 when the anoxic volume was close to the long-term average. Despite large interannual variability, a long-term average of gross phytoplankton production is still an order of magnitude greater than chemoautotrophy (Harding et al. 2002) and, thus, it would not affect our conclusion. Any external source of organic carbon is not included because physical transport of organic carbon can only reach 10.8 mmol m⁻² day⁻¹ (= 3.9 mol m⁻² yr⁻¹ × (365 day)⁻¹) in the mesohaline region of tributaries (Kemp et al. 1997);

further, considering lower river discharge in summer and low biodegradability of terrestrial organic matter, it would contribute less to the summer labile organic pool. Lastly, as only a fraction of dissolved organic carbon is labile (Jonas and Tuttle 1990) and degradable by anaerobic heterotrophs (Bastviken et al. 2003), we estimated extracellularly released organic carbon (ROC) flux using a relationship with phytoplankton production as a proxy of labile dissolved organic carbon (Chrost and Faust 1983; Baines and Pace 1991).

Overall, our results suggest that 5.8 ± 0.8 % of total autochthonous organic carbon production was attributed to chemoautotrophy during summer anoxia (Fig. 7a). The average chemoautotrophic CO₂ fixation rate was about a 3.4-fold and 4.5-fold smaller than ROC and BP, respectively. This suggests a stronger dependence of heterotrophic metabolism on phytoplankton derived organic carbon than chemoautotrophic production.

We also estimated the budget of organic carbon remineralization and fluxes, including aerobic and anaerobic respiration, sediment-water flux, and sediment burial in terms of carbon (Fig. 7b). Anaerobic respiration is the sum of respiration rates measured below oxyclines and the flux of NO₃⁻, NO₂⁻, Mn(IV), and Fe(III) converted to carbon equivalents across the oxyclines, assuming a complete reduction (e.g., NO₃⁻ to N₂) of electron acceptors (Table 2). Burial of organic carbon is assumed to be ~20 % of sediment-water flux according to previous studies (Roden et al. 1995; Kemp et al. 1997). If we assume a quasi-steady state of physical forces in terms of the input/export of organic carbon during seasonal anoxic events, net ecosystem metabolism, estimated by subtracting the loss from the gain of organic carbon flux, resulted in -43.3, -37.8, -4.2, and 44.8 mmol m⁻² day⁻¹ on 24 May, 14 June, 8 July, and 8 August, respectively (Fig. 7b). The magnitude and pattern of ecosystem trophic status from net heterotrophy to autotrophy was almost identical to a previous study that only included

phytoplankton production in the production term (e.g., Kemp et al. 1997). Additionally, considering the high variability of phytoplankton production dynamics related to river discharge (Harding et al. 2002), the fraction of chemoautotrophy to total organic carbon would be even smaller during a year of average river discharge.

5. Conclusion

We assessed the contribution of different modes of primary production and respiration across a gradient of DO. The results suggest that such processes are highly adapted to the change of redox conditions in the estuarine water column. Contrary to the minimal effect of chemoautotrophy on the budget of total organic carbon in the Chesapeake Bay, previous studies reported an important role of chemoautotrophy in different environments (Table 3). For example, DarkPP was as high as the production of OxyPP at oxic/anoxic interfaces in the estuarine turbidity maximum region of the Ebro River where light is major limiting factor for phytoplankton growth (Casamayor et al. 2001). Further, higher contribution of chemoautotrophy than phytoplankton to the carbon budget has been observed in the Cariaco Basin (Taylor et al. 2001) and a monomictic lake (García-Cantizano et al. 2005) due to lateral advection of oxidized waters and reduced sulfur, respectively. Contrary, the bottom boundary of euphotic depths usually located within oxyclines (Fig. 3c, 5c) and lateral (i.e., east/west direction) exchange of bottom waters is topographically restricted in the mesohaline Chesapeake Bay. Therefore, these results along with our data suggest that relative magnitude of chemoautotrophy to total production strongly depends on a gradient of environmental, physical, geochemical, and hydrodynamic characteristics.

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Tables

Table 1. Correlation matrix of biogeochemical values at different depths (lower left) and gradients across neighboring depths (upper right) during the temporal and transect survey (n = 88). Only significant ($p < 0.05$) results are present and bold coefficients indicate $p < 0.001$. Units: density (kg m^{-3} or kg m^{-4}); temperature ($^{\circ}\text{C}$ or $^{\circ}\text{C m}^{-1}$); chlorophyll a and pheophytin ($\mu\text{g L}^{-1}$ or $\mu\text{g L}^{-1} \text{ m}^{-1}$); dissolved oxygen and inorganic compounds ($\mu\text{mol L}^{-1}$ or $\mu\text{mol L}^{-1} \text{ m}^{-1}$).

Abbreviations: Temp: temperature; Chl-a: chlorophyll a; Pheo: pheophytin; DO: dissolved oxygen; SRP: soluble reactive phosphorus; DS: total dissolved sulfide; DFe: dissolved iron; DMn: dissolved manganese.

	Density	Temp	Chl-a	Pheo	DO	NO_2^-	NO_3^-	NH_4^+	SRP	DS	DFe	DMn
Density		-0.79	-	-	-0.65	-	-	0.71	-	0.29	-	0.51
Temp	-		-	-	0.73	-	-	-0.86	-	-0.32	-	-0.63
Chl-a	-0.55	-		0.58	0.42	-0.33	-	-	0.27	-	-	-
Pheo	-0.42	-0.26	0.70		0.24	-	-	-	-	-	-	-
DO	-0.74	-	0.69	0.55		-0.32	-	-0.69	-	-	-	-0.39
NO_2^-	-	-	-	-	-		-	-	-0.68	-	-	-
NO_3^-	-0.35	-0.56	-	0.28	0.49	0.31		-	-	-	-	-
NH_4^+	0.58	-0.23	-0.45	-0.33	-0.76	-0.28	-0.34		-	0.35	-	0.70
SRP	0.39	-	-	-0.31	-0.38	-0.25	-0.36	0.53		0.23	-	0.28
DS	0.43	-	-0.27	-0.32	-0.47	-0.25	-0.23	0.70	0.53		-	0.33
DFe	-	-	-	-	-	-0.29	-	-	-	-		0.49
DMn	0.35	-	-0.31	-0.36	-0.60	-	-0.25	0.84	0.50	0.61	0.32	

Table 2. The flux of electron acceptors and donors in terms of mass ($\text{mmol m}^{-2} \text{ day}^{-1}$) and electron equivalent ($\text{mmol e}^{-} \text{ m}^{-2} \text{ day}^{-1}$) across oxyclines. The number of transferable electron (e^{-}) is based on the assumption of complete reduction and oxidation of respective electron acceptors and donors.

Date		Electron acceptor					Electron donor				Balance
		DO	NO_3^{-}	NO_2^{-}	Mn(IV)	Fe(III)	DS	NH_4^{+}	DMn	DFe	
	Transferable e^{-}	4	5	3	2	1	8	8	2	1	
24 May	Mass flux	-46.8	-2.8	-0.2	-0.4	-0.1	0.0	6.8	0.4	0.1	
	Electron flux	-187.3	-13.9	-0.5	-0.9	-0.1	0.0	54.1	0.9	0.1	-147.5
14 June	Mass flux	-13.1	-0.5	-0.5	-0.5	-0.2	1.3	4.1	0.5	0.2	
	Electron flux	-52.2	-2.3	-1.6	-1.0	-0.2	10.3	33.1	1.0	0.2	-12.7
8 July	Mass flux	-31.3	0.0	0.0	-1.7	-0.7	7.6	6.5	1.7	0.7	
	Electron flux	-125.1	-0.1	0.0	-3.4	-0.7	60.6	51.6	3.4	0.7	-13.0
8 August	Mass flux	-62.1	0.0	0.0	-1.8	-2.3	4.8	7.6	1.8	2.3	
	Electron flux	-248.3	-0.2	0.0	-3.7	-2.3	38.1	60.5	3.7	2.3	-150.0

Table 3. Depth-integrated chemoautotrophy in different ecosystems and a comparison with gross phytoplankton production (GPP).

	Oxic/anoxic interface interval	Chemoautotrophy	Contribution to GPP	
System	(m)	(mmol m ⁻² day ⁻¹)	(%)	Source
Ebro River estuary	3-5	3.5	48.0 ^a	Casamayor et al. 2001
Ciso´ Lake	0.5-1.5	28.8	52.0	García-Cantizano et al. 2005
Cadagno Lake	10.8-11.4	130.0	50.0	Camacho et al. 2001
Big Soda Lake	3-4	31.5	30.0	Cloern et al. 1983
Cariaco Basin	80-100	82.0	62.0	Taylor et al. 2001
Chesapeake Bay	8.5-10.1	14.0	5.8 ± 0.8	This study
^a : Within the oxic/anoxic interface				

Figures Legends

(Fig. 2, 3, and 5 are intended for color production on the Web and black-and-white in print.)

Fig. 1. The map of Chesapeake Bay with a depth gradient. The stations were located along a shipping channel and stretched from near the mouth of Potomac River to the Chesapeake Bay Bridge.

Fig. 2. Density (top) and natural log-transformed buoyancy frequency (bottom) during the temporal (a, b) and transect (c, d) survey. Gray asterisk represents sampling depth during the temporal survey. During the transect survey, asterisk is omitted because undulating towed vehicle was used. Gray area represents bottom topography.

Fig. 3. Temporal transition of biogeochemical variables reflecting redox conditions in the mesohaline Chesapeake Bay. Note that the survey was conducted at single station (S3) for seven times. Asterisk represents sampling depth. Dotted line on the map of chlorophyll a represents the depth of 1% of surface irradiance. (DIC: dissolved inorganic carbon; SRP: soluble reactive phosphorus; DS: dissolved sulfide; DM: dissolved manganese; DFe: dissolved iron).

Fig. 4. Respiration and primary production in dark at S3 during the temporal survey. Horizontal dotted and dash line represents 2 and 0.5 mg O₂ L⁻¹ isopleth, respectively. Error bar represents standard error of triplicate samples.

Fig. 5. Spatial transition of biogeochemical variables reflecting redox conditions along the main axis of Chesapeake Bay. Asterisk represents sampling depth. Dotted line on the map of chlorophyll a represents the depth of 1% of surface irradiance. Gray area represents bottom topography. The scale for each variable is identical to that in Fig. 3.

Fig. 6. Respiration and dark primary production at five stations during the transect survey.

Horizontal dotted and dash line represents 2 and 0.5 mg O₂ L⁻¹ isopleth, respectively. Error bar represents standard error of triplicate samples.

Fig. 7. (a) Percentage contribution of gross phytoplankton production (GPP), extracellularly released organic carbon (ROC), and chemoautotrophy (DarkPP) to total organic carbon production in the mainstem Chesapeake Bay. (b) Percentage contribution of aerobic respiration (Aerobic R.), anaerobic respiration (Anaerobic R.), sediment-water DIC flux (Sed. flux), and sediment burial to total organic carbon loss. The line graph represents the sum of absolute values in (a), while the dashed graph represents the sum of absolute values in (b); both graphs use a secondary y-axis.

Figures

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7