

ENVIRONMENTAL STUDIES

Correcting a major error in assessing organic carbon pollution in natural waters

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Microbial degradation of dissolved organic carbon (DOC) in aquatic environments can cause oxygen depletion, water acidification, and CO₂ emissions. These problems are caused by labile DOC (LDOC) and not refractory DOC (RDOC) that resists degradation and is thus a carbon sink. For nearly a century, chemical oxygen demand (COD) has been widely used for assessment of organic pollution in aquatic systems. Here, we show through a multicountry survey and experimental studies that COD is not an appropriate proxy of microbial degradability of organic matter because it oxidizes both LDOC and RDOC, and the latter contributes up to 90% of DOC in high-latitude forested areas. Hence, COD measurements do not provide appropriate scientific information on organic pollution in natural waters and can mislead environmental policies. We propose the replacement of the COD method with an optode-based biological oxygen demand method to accurately and efficiently assess organic pollution in natural aquatic environments.

INTRODUCTION

About one-quarter of the net carbon fixation on land enters the dissolved organic carbon (DOC) pool of natural waters (1–3). The nature and behavior of DOC in aquatic systems determine its fate and environmental effects. DOC is often classified based on how rapidly it turns over in the natural environment (4). Labile DOC (LDOC) is rapidly degraded and taken up by microbes, and when high amounts of LDOC are released into the environment, the resulting consumption of oxygen can cause oxygen depletion (5, 6) with detrimental effects on aquatic ecosystems. Also, respiration of LDOC to CO₂ can result in water

acidification (6, 7) and release of excess CO₂ (supersaturation) to the atmosphere where it contributes to the greenhouse effect. In this way, a productive coastal region could become a source rather than a sink of atmospheric CO₂ (7, 8). It follows that the microbial degradation of LDOC can lead to a series of environmental problems. In contrast, the refractory components of DOC (RDOC) in the aquatic environment are not readily or rapidly metabolized by microbes and so can be preserved in natural waters where they act as a carbon sink (4, 9). While RDOC includes chelating agents (10) and therefore may act as an important transport pathway or sink for heavy metals, RDOC is not a pollutant but contributes instead to carbon sequestration. RDOC accounts for more than 95% of the total DOC in the deep ocean (4, 11) (man-made persistent organic pollutants are not discussed in this paper because they represent very small amounts of the DOC; Supplementary Materials, statement 1.1). A purely chemical view of organic pollution could identify both LDOC and RDOC as organic pollutants, whereas the ecological view of organic pollution clearly restricts pollutants to LDOC. The negative environmental consequences of the microbial degradation of organic compounds mentioned above (i.e., oxygen depletion, acidification, and release of CO₂ to the atmosphere) depend only on the magnitude of the LDOC fraction.

The concern generated by organic pollution led governments to monitor microbial degradability of organic matter in water bodies, which became a fundamental tool in environmental policy and management. The biodegradability of organic matter can be evaluated via its oxygen demand by microorganisms, i.e., the amount of oxygen consumed by bacteria and other microorganisms while they decompose and metabolize the organic matter under aerobic conditions. The oxygen demand of LDOC in aquatic environments is best measured as the biological (or biochemical) oxygen demand (BOD) (12). However, the existing standard BOD method is time consuming as it requires 5-day incubations, and has low reproducibility as it involves initial and final measurements on different subsamples

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(bottles) of the water samples (12). Consequently, BOD estimates have largely been replaced by measurements of chemical oxygen demand (COD), i.e., the determination of the amount of oxygen consumed by chemical reactions in the solution. In COD, potassium dichromate or potassium permanganate is used to rapidly oxidize the organic matter (and also possibly existing reductive inorganic ions) by chemical reactions, thus shortening the measurement time to only a few hours. Given its convenience, relative simplicity, and repeatability, the COD method has been widely used by agencies and scientists for nearly a century for monitoring and managing waste effluents and natural waters (Supplementary Materials, statements 1.2 and 1.3) (13). However, the COD method requires the addition of high amounts of strong chemical oxidants (i.e., potassium dichromate and permanganate), which do not exist in natural waters. Hence, the COD method artificially oxidizes both LDOC and much or all of the RDOC, whereas oxygen consumption in natural waters is only caused by LDOC oxidation. Although the use of the COD method is justified when rapid measurements are required, such as in the management of wastewater treatment plants, we question its scientific basis and effectiveness for the assessment of organic pollution in natural water bodies.

Here, we hypothesize that the COD method substantially overestimates the DOC that can be degraded by microbes in natural waters, with the consequence that using this method could mislead the assessment of organic pollution in aquatic environments (Fig. 1).

To test our hypothesis, we analyzed COD and BOD data from experiments conducted for this study and measurements collated from the literature (see Materials and Methods). These data cover a wide variety of natural aquatic environments ranging from tropical to subarctic zones, farmlands to forest watersheds, high plateaus to coastal waters, and fresh waters to saline lakes and marine waters (fig. S1 and table S1). We thus examined how COD and BOD vary across gradients of salinity and DOC with latitude and between ecosystems.

Two methods are currently used to determine COD, based on potassium dichromate (COD_{Cr}) and potassium permanganate oxidation (COD_{Mn}) (see Materials and Methods) (14). Comparison of COD_{Cr} and COD_{Mn} values determined on natural waters of different salinities and DOC concentrations ([DOC]) indicated that COD_{Cr} is not a valid measure of oxidized organic matter because salinity interferes with the determination of [DOC] [salinity effect already reported in previous publications including (14)], whereas COD_{Mn} may provide appropriate measurements (figs. S2 and S3 and tables S2 and S3). Hence, we only use COD_{Mn} values in the following analysis. To simplify terminology, we use “COD” instead of “ COD_{Mn} ” hereinafter and “DOC” instead of total organic carbon (TOC) for the following discussion (Supplementary Materials, statement 1.4).

We also used a water-column macrocosm for a long-term incubation of natural water to better understand the problems involved in the current COD method and the chemical nature of the organic matter oxidized by this method. We incubated in the Aquatron Tower Tank of Dalhousie University 100 m³ of natural river water rich in humic material in darkness for 510 days (see Materials and Methods). The DOC in the incubated water was therefore subjected to microbial degradation for a much longer time than the 5 days required by the BOD method. Operationally speaking, any organic matter remaining after the 510-day incubation would have been refractory. We used, for the first time in the literature, ultrahigh-resolution Fourier transform ion cyclotron resonance mass

spectrometry (FT-ICR MS) to analyze the water samples subjected to different treatments regarding the COD methodology (see Materials and Methods). A water sample from 1000-m depth in the South China Sea was used as a reference for natural-seawater RDOC.

RESULTS AND DISCUSSION

Investigating the COD problems with field data

Data from our samples and the literature (fig. S1 and table S1) show that the relationships of COD and BOD with [DOC] vary in different natural environments (Fig. 2). The COD values increase linearly with increasing [DOC] in all the sampled natural environments, indicating that COD oxidizes both LDOC and RDOC, as expected. In contrast, the BOD values are much lower than their COD counterparts, as they reflect only LDOC. The relationships observed between BOD and [DOC] across different environments and the variation therein can be explained by our current understanding of organic matter cycling as follows.

First, we explored latitudinal differences in COD and BOD as a function of [DOC] by grouping the data into high latitudes (subpolar areas), mid-latitudes (temperate zone), and low latitudes (subtropical zone) (Fig. 2, A to C). While the relationships of COD and BOD with [DOC] are totally decoupled at high latitudes (Fig. 2A), they are better aligned at lower latitudes (Fig. 2, B and C). In the subpolar environment, the low BOD values and their independence from [DOC] (low correlation and regression coefficients, i.e., 0.16 and 0.39, respectively) indicate generally low LDOC concentration, while the increase of COD-BOD with increasing [DOC] indicates accumulation of RDOC with increasing [DOC]. In the temperate zone, the values of COD and BOD both increase with [DOC], and the COD-BOD values are generally similar to the BOD values. This indicates similar concentrations of LDOC and RDOC at mid-latitudes. In the subtropical zone, the COD-BOD values are lower than BOD, indicating that COD is dominated by LDOC with less RDOC accumulating in the environment than at mid-latitudes (Fig. 2, B and C). In terms of the absolute value of COD, the subpolar zone ranks the highest, followed by the temperate and subtropical zones, the latter being the lowest, whereas the BOD values remain at the same general level (Fig. 2). If these COD values were assessed according to conventions used in environmental management, the high COD values at high latitude would be interpreted as highly organic polluted compared with low latitudes. Instead, the high COD values are due to high concentrations of nonpolluting RDOC (see above).

Second, we grouped the same samples as in Fig. 2 (A to C) by ecosystems (Fig. 2, D to F). This led to the emergence of distinct patterns in LDOC and RDOC that reflect changes in organic matter as it moves through the hydrological cycle toward the sea. In waters of forested regions, BOD is almost independent of [DOC], and COD-BOD increased proportionally with [DOC]. The latter is consistent with DOC in forested watersheds consisting mostly of humic-like compounds, which are common components of RDOC (15, 16). In the other freshwater samples (Fig. 2E), the linear relations of COD-BOD and BOD with [DOC] were similar, indicating similar fractions of LDOC and RDOC in DOC. In coastal water samples, the values of BOD are higher than those of COD-BOD, indicating that COD is dominated by LDOC. If the conventional COD standards were applied to the assessment of water quality in these different ecosystems, waters of forested regions would be considered as loaded with organic pollutants. Instead, the high COD values are due to

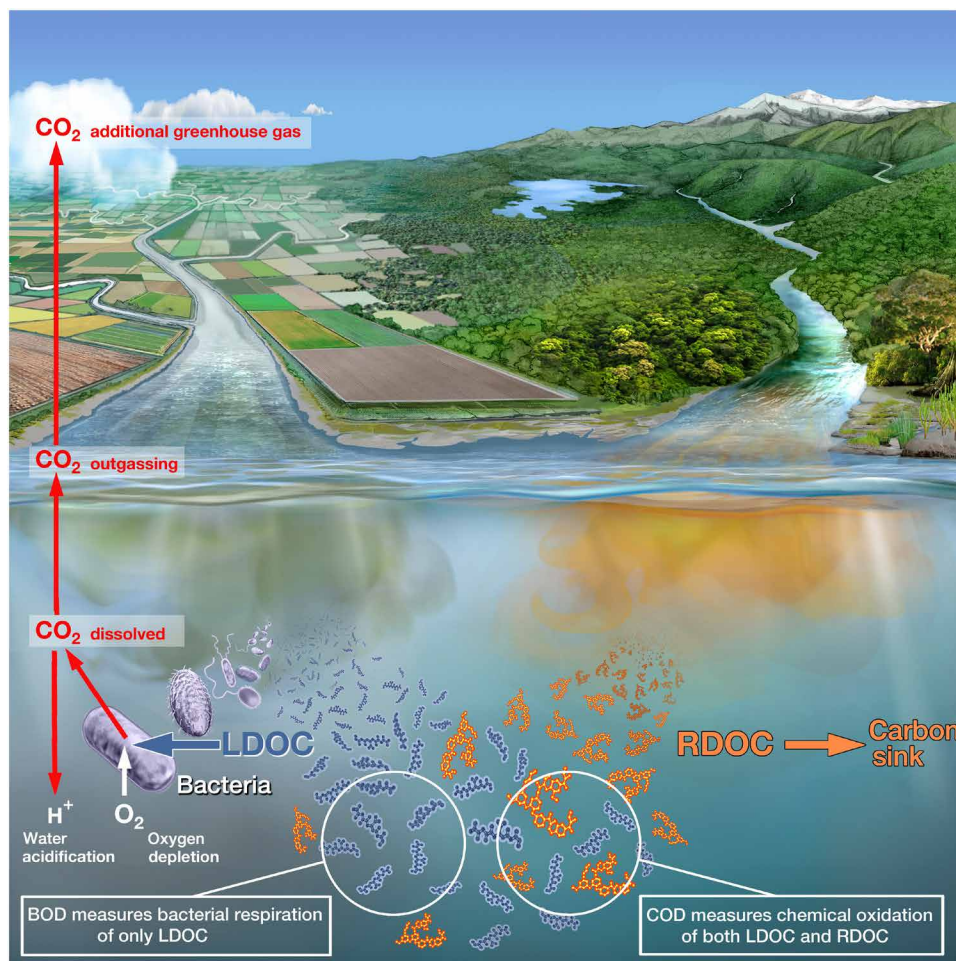


Fig. 1. Schematic representation of LDOC and RDOC in the environment and their determination using the BOD and COD approaches. Degradation of LDOC can cause oxygen depletion, water acidification, and CO₂ emission and is best assessed by the BOD method. Only LDOC is readily degradable by microbes in natural waters; RDOC has no adverse environmental effects and is a carbon sink. The widely used COD method oxidizes both LDOC and RDOC and so could result in a major error in the assessment of organic pollution.

high concentrations of nonpolluting humic-like RDOC compounds (see above). This shows again the drawback of relying on COD as an indicator of organic pollution in natural aquatic systems.

Together, the above field observations are consistent with our hypothesis that COD reflects the oxidation of both LDOC, which can be naturally degraded by microbes, and RDOC, which resists biodegradation. In contrast, BOD reflects the oxidation of LDOC only. Hence, COD is not a valid indicator of the microbial degradability of organic compounds, and thus organic pollution in aquatic environments, whereas BOD provides realistic estimates of biodegradability.

Investigating the COD problems experimentally

To further investigate which fractions of naturally occurring organic matter are oxidized by the COD method, samples from the 510-day incubation in the Aquatron Tower Tank were treated with or without the COD method, and FT-ICR MS analyses were used to identify differences between treatments. Results are summarized in Fig. 3, where a water sample from 1000-m depth in the South China Sea provides a reference for natural-seawater RDOC (Fig. 3G) (17). The

FT-ICR MS raw peak distributions [mass/charge ratio (m/z) 200 to 600] showed no major changes in components of the samples before and after the 510-day incubation (Fig. 3, A and B, respectively), reflecting the natural richness of the river water in RDOC. In contrast, there was a large change in the FT-ICR MS peak distributions after the COD treatment of both the initial and 510-day incubated samples (Fig. 3, C and D). This shows that most DOC molecules in the m/z range of 200 to 600 persisted during the 510-day incubation (Fig. 3, A and B) but were oxidized by the COD treatment of both the initial and incubated water (Fig. 3, A versus C and B versus D).

The FT-ICR MS analysis provides a proxy for the naturally occurring RDOC, i.e., the polygons in Fig. 3 (E to G) delineate carboxyl-rich alicyclic-like (CRAM-like) molecules based on the literature (10, 18). These molecules dominate the DOC of the deep ocean, where they are identified as RDOC (10, 17, 19). Figure 3 (E and F) shows the molecules that were oxidized by the COD method (i.e., samples in Fig. 3, A minus C and B minus D, respectively). A large part of the oxidized molecules corresponds to the naturally occurring CRAM-like molecules in the deep seawater (Fig. 3G). These

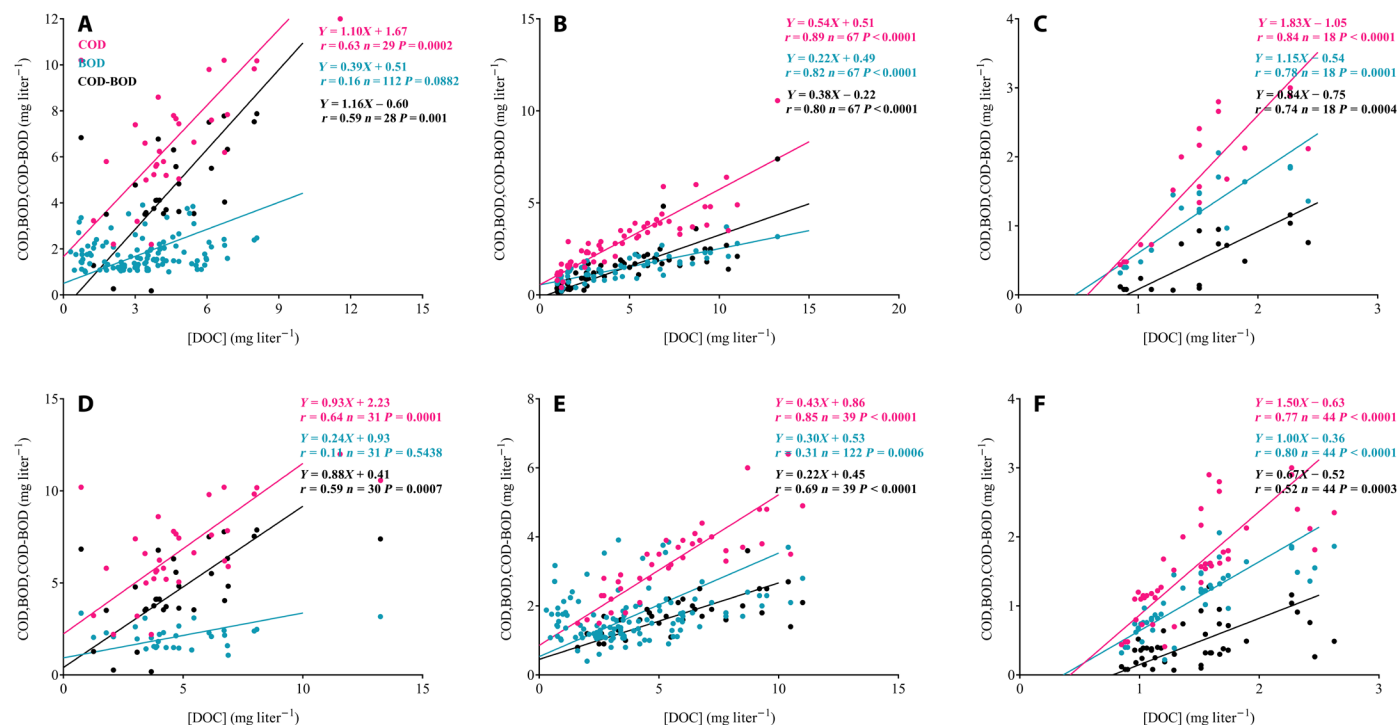


Fig. 2. COD and BOD as a function of [DOC] in different environments. (A) High latitudes/subpolar areas. (B) Mid-latitudes/temperate zone. (C) Low latitudes/subtropical zone. (D) Forested watersheds. (E) Fresh waters. (F) Seawater. Note the different x- and y-axis scales of the different panels. The same samples were grouped by (A to C) latitudes and also (D to F) ecosystems. Regressions: model 2 standard major axis. n : number of samples.

results demonstrate that the COD treatment rapidly oxidizes RDOC molecules that would otherwise persist for long periods as part of the carbon sink in natural waters.

Resolving paradoxical results arising from monitoring pollution as COD

In the practical application of COD methods in natural waters, there is often a paradoxical decoupling between COD and BOD, including in situations where environmental protection measures have been carried out for decades. Representative examples are Lake Biwa in Japan (20), the Han River in Korea (21), and Finnish rivers (22) (Fig. 4). Lake Biwa was notorious for its organic pollution in the 1970s, but this pollution was reduced over the past three decades. It might therefore be expected that COD, as an indicator of organic pollution, would have decreased in response to the abatement of organic pollution. However, COD has increased since the 1980s, whereas BOD has decreased (Fig. 4A). This apparent paradox can be explained by a long-term accumulation of RDOC in the lake, which can be oxidized by the COD but not the BOD method. In cases such as Lake Biwa, environmental protection agencies should definitely use BOD instead of COD for assessing water quality. In the Han River (Fig. 4B), the COD values remained fairly constant over 20 years, whereas the BOD values continuously decreased. This means that the relative fraction of RDOC increased, while the LDOC fraction decreased over the past 20 years along with less eutrophication that would remobilize RDOC for decomposition (23). In Finnish rivers (Fig. 4C), COD and BOD both decreased over the past decades, and since BOD decreased faster than COD, the COD-BOD/

COD ratio increased. This indicates that the relative fraction of RDOC increased and that of LDOC decreased.

Again, these long-term observations show that COD measurements on their own do not provide consistent estimates of organic pollution, whereas the BOD values yield these estimates in all circumstances. Furthermore, COD data used alone may fail to detect, or mask, the success of environmental protection countermeasures at curbing organic pollution.

Replacing COD by a modern BOD method

The above analyses show that COD is not a valid indicator of the microbial degradability of organic compounds and thus organic pollution in natural waters. This is because COD reflects the oxygen demand of both LDOC and RDOC, and the latter is not, or at least is very inefficiently, oxidizable by microbes and is thus part of the carbon sink. The fact that COD confounds RDOC with organic pollution implies that it can mislead environmental assessments or the application of environmental policies. However, COD is currently used widely for the monitoring and management of natural waters (13, 24–26). As explained above, the present study has no bearing on the use of COD in wastewater treatment plants but addresses its use with natural waters. Many governmental and international organizations presently rely on COD data to establish environmental standards and policies (Supplementary Materials, statement 1.2 and tables S4 and S5). Hence, there is a pressing need to prevent the misinterpretation created by the use of COD, especially considering that the International Organization for Standardization recommended COD as one of the parameters for assessing the quality of natural waters (27). Many countries use COD as the primary standard

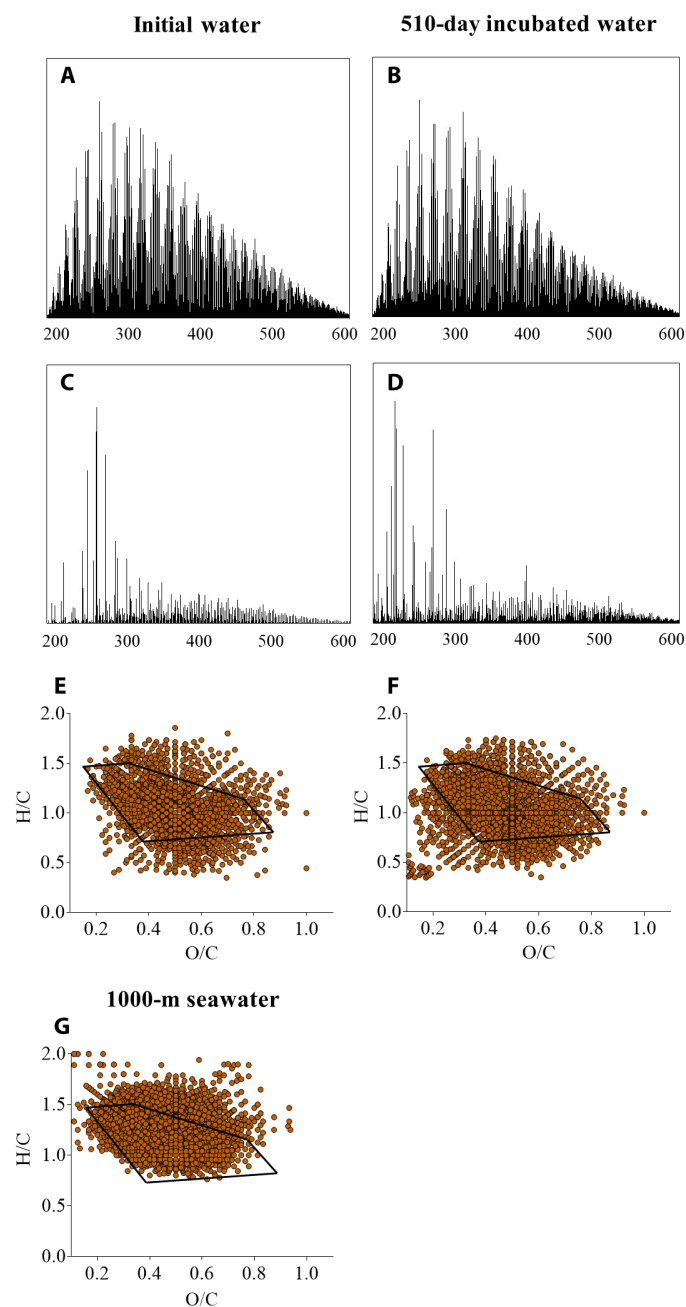


Fig. 3. Results of FT-ICR MS analyses showing changes in organic matter in the natural and incubated river water samples before and after COD treatment. (A and B) Raw peak distributions (m/z 200 to 600) of organic matter in the water samples before and after long-term (510 days) incubation, respectively, showing no major differences before and after long-term microbial degradation. (C and D) Raw peak distributions (m/z 200 to 600) of remaining organic matter after the COD treatment of the same samples as in (A) and (B), respectively. (E and F) van Krevelen diagrams of the RDOC-like components oxidized by the COD treatment, i.e., samples in (A) minus (C) and (B) minus (D), respectively. (G) Deep-water (1000-m) sample from the South China Sea, used as an RDOC reference. The black polygons in (E) to (G) delineate the CRAM-like molecules (RDOC proxy), based on the literature.

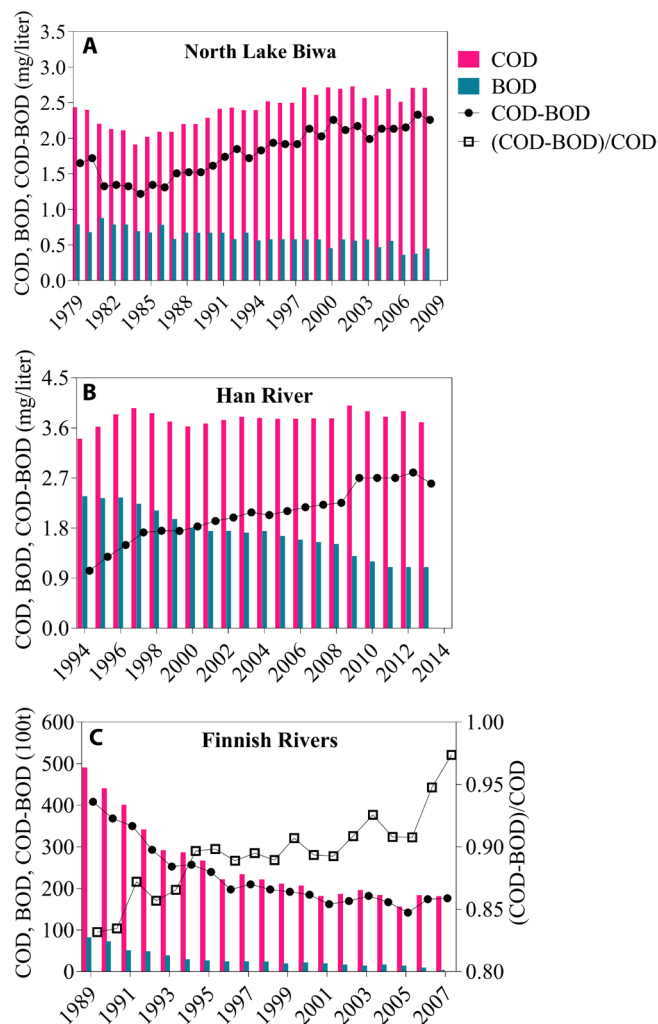


Fig. 4. Three representative field cases of long-term trends in COD and BOD changes. (A) Increase in COD, decrease in BOD, and thus accumulation of RDOC (as indicated by [COD-BOD]) in Lake Biwa, Japan. (B) Sharp decrease in BOD, relatively stable COD, and thus accumulation of RDOC in the Han River, Korea. (C) Decrease in both BOD and COD but increase in the fraction of RDOC in Finnish rivers.

for water quality control and environmental assessment. For example, in China and Japan, the performance of environmental policies has been assessed on the basis of the reduction of COD (tables S4 and S5).

A substitute for the COD method is therefore required. TOC has been proposed for the monitoring of wastewater treatment and aquatic environments (13, 28). However, TOC measurements include RDOC, which disqualifies it as an appropriate measurement to determine the microbial degradability of organic compounds. Similarly, total oxygen demand (29) is not an appropriate alternative to COD as it incorporates the oxidation of RDOC. In contrast, BOD has clear scientific basis and remains the first choice as an alternative to COD. However, the traditional BOD method has been abandoned in many instances because of its labor-intensive nature and also the variability introduced by the heterogeneity among the bottles used for oxygen titrations before and after incubation. Here, we recommend a modern BOD method that uses oxygen optodes (30, 31) instead of oxygen titration for the measurement of oxygen concentration, where the latter can be conducted in the same incubated bottles before and

after incubation (or even continuously). This reduces the number of incubation bottles, reduces procedural errors, and eliminates the use of hazardous chemicals (see Materials and Methods). The optode-based BOD measurements provide repeatable values that are more indicative of water quality than COD (figs. S4 and S5).

From field measurements to environmental policy

The above results of our multicountry field survey and experimental studies are consistent with our hypothesis that the COD method substantially overestimates the DOC that can be degraded by microbes in natural waters, showing that the application of the COD method often misleads the assessment of organic pollution in aquatic environments. We recommend the modern optode-based BOD method to replace the COD method for application in natural aquatic environments. These measurements are required to inform policy makers of the risks of adverse environmental conditions that can be triggered by high concentrations of LDOC and to design and implement measures to improve water quality. With the increasing need to monitor and manage water quality, it is imperative to refine and improve the scientific measurements on which policy depends.

MATERIALS AND METHODS

Sampling sites and data from the literature

The field data used in this paper include new measurements made for this study and data collated from the literature. The field measurements were conducted in China (coastal water samples from the Xiamen Bay, Sanya Bay, Bohai Sea, and Yellow Sea and freshwater samples from Fujian, Sichuan, and the Tibetan Plateau), Canada (Nova Scotia, New Brunswick, Quebec, and British Columbia), and the United States (Florida). The sampling locations are shown in fig. S1, and detailed information is provided in table S1. To keep the data comparable, we only took from the literature COD values measured using the titration COD_{Mn} method and BOD data using the BOD₅ method (described below) (20–22, 32–35).

COD_{Cr} method

There are two methods for COD measurements: one with potassium dichromate as oxidant (COD_{Cr}) and the other with potassium permanganate (COD_{Mn}, which has two variants or protocols described in the next section). The measurement of COD_{Cr} was based on the International Standard ISO6060 “Determination of Chemical Oxygen Demand in Water” (www.iso.org/standard/12260.html), which was officially reviewed and confirmed in 2017. Briefly, 10 ml of a water sample was added into a 250-ml conical flask. Five milliliters of K₂Cr₂O₇ solution was added into the water and thoroughly mixed. After connecting the conical flask into a condensing system, 15 ml of AgSO₄-H₂SO₄ solution was slowly added into the mixture, which was then heated to boiling point for 2 hours. After that, about 45 ml of ultrapure water was added. Cooling to room temperature, one or two drops of ferroin were added into the mixture to serve as a titration indicator, and titration was conducted with (NH₄)₂Fe(SO₄)₂·6H₂O solution and ended after the color of the mixture turned brown. The consumed volume of (NH₄)₂Fe(SO₄)₂·6H₂O solution was recorded as V₁ (ml). As a control, 10 ml of ultrapure water was used for the titration described above, and the consumed volume of the (NH₄)₂Fe(SO₄)₂·6H₂O solution was recorded as V₀ (ml). COD_{Cr} (mg O₂ liter⁻¹) was calculated from the following equation

$$\text{COD}_{\text{Cr}} = (c((\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}) \times (V_0 - V_1) \times 8000)/10$$

Triplicate water samples were analyzed at each station.

COD_{Mn} protocols

There are two COD_{Mn} protocols in the literature: alkaline COD_{Mn} and acidic COD_{Mn} protocols. The alkaline COD_{Mn} protocol is described in the Chinese national standard method of COD for seawater (GB1737.4-2007) and uses KMnO₄ as oxidant in alkaline conditions. Briefly, a 100-ml water sample was added into a 250-ml conical flask. One milliliter of NaOH (250 g liter⁻¹) and 10 ml of KMnO₄ solution (0.01 M) were added to the water and thoroughly mixed. The mixture was heated to boil for 10 min, after which it was quickly cooled to room temperature. After this, 5 ml of H₂SO₄ solution (5 M) and 0.5 g of KI were added to the cooled mixture, which was then placed in the dark for 5 min. With the solution continuously shaken, titration was conducted with Na₂S₂O₃·5H₂O solution (*c* ≈ 0.01 M, calibrated by KIO₃ standard solution) until the color of the mixture turned light yellow. Then, 1 ml of starch solution (5 g liter⁻¹) was added to the mixture and the titration was continued until the mixture turned transparent. The consumed volume of the Na₂S₂O₃·5H₂O solution was recorded as V₁ (ml). As a control, 100 ml of ultrapure water was used for the titration described above, and the consumed volume of the Na₂S₂O₃·5H₂O solution was recorded as V₀ (ml). Triplicate water samples were analyzed at each station. COD_{Mn} (mg O₂ liter⁻¹) was calculated using the following equation

$$\text{Alkaline COD}_{\text{Mn}} = (c(\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}) \times (V_0 - V_1) \times 8000)/100$$

The acidic COD_{Mn} protocol is described in the Chinese national standard method of COD for surface water (GB11892-89) and uses KMnO₄ as oxidant in acidic conditions. Briefly, a 100-ml water sample was added into a 250-ml conical flask. Five milliliters of H₂SO₄ solution (5 M) and 10 ml of KMnO₄ solution (0.01 M) were added to the water and evenly mixed. The mixture was heated for 30 min in a boiling water bath. Ten milliliters of Na₂C₂O₄ solution (0.01 M) was then added to the mixture, which turned transparent. With the solution continuously shaken, the titration was conducted with KMnO₄ solution (0.01 M) until the color of the mixture turned pink and remained pink for at least 30 s. The consumed volume of the KMnO₄ solution was recorded as V₁ (ml). As a control, 100 ml of ultrapure water was used for the titration described above, 10 ml of Na₂C₂O₄ solution (0.01 M) was added to the mixture, KMnO₄ solution was used as the titrant, and the consumed volume was recorded as V₀ (ml). Triplicate water samples were analyzed at each station. Acidic COD_{Mn} (mg O₂ liter⁻¹) was calculated using the following equation

$$\text{Acidic COD}_{\text{Mn}} = (((10 + V_1) \times 10/V_0) - 10) \times c(\text{KMnO}_4) \times 8000/100$$

The acidic COD_{Mn} protocol is recognized to be applicable for fresh water but not saline water because of a salinity effect, i.e., an overestimation due to interference of inorganic ions (36–38). We investigated this by comparing results from the acidic and alkaline protocols for samples with salinities ranging from 0 to 30 (table S3). The data from the acidic COD_{Mn} protocol were systematically larger than those obtained with the alkaline COD_{Mn} protocol for salinities >0.3, and the difference increased with salinity (fig. S3). In fresh waters (salinities ≤0.3), some data from the acidic COD_{Mn} protocol were smaller than those from the alkaline COD_{Mn} protocol.

Because both methods use excessive amounts of oxidants, they are unlikely to produce underestimated values. Given that the data in this study came from freshwater, estuarine, and marine environments, we decided to use only the values obtained with the alkaline COD_{Mn} protocol to avoid overestimation of COD values in estuarine and saline waters.

Comparison of COD_{Cr} and alkaline COD_{Mn} measurements

The COD_{Cr} and alkaline COD_{Mn} measurements of natural water samples with different salinities showed that the COD values from the two methods were not influenced in the same way by the presence of inorganic reductants (table S2). The values of COD_{Cr} were much higher than those of COD_{Mn} in all samples, the former being up to 80 times the latter in saline waters (fig. S2A). In addition, the coefficients of correlation of COD_{Cr} and COD_{Mn} with salinity ($r = 0.94$, prob. < 0.001 and $r = 0.54$, prob. = 0.075, respectively) indicate a significant positive relationship between COD_{Cr} and salinity and no relationship between COD_{Mn} and salinity. As a consequence, the ratio COD_{Cr}/COD_{Mn} significantly increased with salinity (fig. S2B). These results show that salinity had a systematic effect on COD_{Cr} measurements.

It follows that the COD_{Cr} does not provide a proper measure of oxidized organic matter especially in saline waters, whereas alkaline COD_{Mn} may provide realistic estimates of oxidized DOC. Hence, to make all our data comparable, we only used COD_{Mn} measurements, either made for this study or collated from the literature (as listed in table S1). To simplify terminology, we use “COD” instead of “alkaline COD_{Mn}” in this paper.

Protocol of BOD by the BOD₅ Winkler method

Five-day BOD (BOD₅) measurement of oxygen consumption by Winkler titration is a standard method by the International Organization for Standardization. Water samples for BOD measurements were incubated in the dark at 20°C for 5 days. The measurement of dissolved oxygen ([O₂]) was based on the ISO 5813:1983 Determination of dissolved oxygen-Iodometric (Winkler) method (www.iso.org/obp/ui/#iso:std:iso:5813:ed-1:v1:en). Briefly, to determine the initial oxygen concentration, 1 ml of MnCl₂ solution (420 g liter⁻¹) and 1 ml of alkaline KI solution (150 g liter⁻¹) were sequentially added into triplicate water samples in 140-ml opaque BOD bottles and then mixed thoroughly, and the samples were allowed to sit in a water bath held at 20°C for at least 4 hours. Thereafter, the precipitate was dissolved with 2 ml of H₂SO₄ solution (5 M). After transferring 100 ml of the solution into a 250-ml conical flask, the titration was conducted as described for the COD_{Mn} method, and the titrated volume of Na₂S₂O₃•5H₂O solution (c , M) was recorded as V_0 (ml). After the 5-day incubation, triplicate water samples were collected and treated by the same procedure as described above, and the titrated volume of Na₂S₂O₃•5H₂O solution was recorded as V_5 (ml). BOD₅ (mg O₂ liter⁻¹) was calculated using the following equation

$$\text{BOD}_5 = c(\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}) \times (V_0 - V_5) \times 1000 \times 8/100$$

For calibration of concentration of Na₂S₂O₃•5H₂O standard solution, 10 ml of KIO₃ standard solution (0.0100 M) was poured into a 250-ml conical flask, and 0.5 g of KI and 1 ml of H₂SO₄ solution (5 M) were sequentially added to the flask, which was then sealed and gently shaken. After keeping the mixture in the dark for 2 min, 50 ml of

ultrapure water was added and gently mixed, in preparation for titration. The titration was conducted with a Na₂S₂O₃•5H₂O solution until the color of the mixture turned light yellow, after which 1 ml of starch solution (5 g liter⁻¹) was added, and the titration was continued until the mixture became transparent. The consumed volume of the Na₂S₂O₃•5H₂O solution was recorded as V (ml). The concentration of the Na₂S₂O₃•5H₂O solution (M) was calculated using the following equation

$$c(\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}) = 10 \times 0.0100/V$$

Determination of DOC

Samples for DOC measurements were filtered through precombusted (450°C, 4 hours) Whatman GF/F glass fiber filters. All the samples were then acidified to pH 2 with H₃PO₄ and stored at -20°C until analyzed. The carbon content of samples was measured using the high-temperature combustion method with a Shimadzu TOC-LCPH TOC analyzer and ultrapure water for system blank subtraction (39). Reference deep seawater (provided by the Hansell Organic Biogeochemistry Laboratory at the University of Miami, USA) served as an additional control (40). The analytical methodology for DOC was the same for all studies reported in our work.

Aquatron Tower Tank and long-term incubation experiments

Located at Dalhousie University (Halifax, Canada), the Aquatron is the site of the Tower Tank, which is 10.64 m deep and 3.66 m in diameter with an approximate water volume of 117 m³ (www.dal.ca/faculty/science/biology/research/facilities/aquatron.html). It is well suited for biogeochemical research requiring depth and stratification. To conduct our long-term incubation experiment, we filled the Tower Tank with about 20 tons of humic-like water from the Ingramport River near Halifax. The incubation was run at room temperature (20°C) in the dark for 510 days. Water samples were taken before and after the incubation and filtered through Whatman GF/F filters (precombusted as above) for solid-phase extraction of dissolved organic matter (DOM) (see below).

Solid-phase extraction of natural DOM- and COD-treated DOM and FT-ICR MS analysis

DOM was solid-phase extracted following a standard procedure (41). For the initial (DOC concentration = 885.96 ± 6.60 μmol C liter⁻¹) and long-term incubation (510-day) (DOC concentration = 744.06 ± 6.80 μmol C liter⁻¹), DOM was extracted from water samples using 500 mg of Bond Elut-PPL cartridges (Agilent) activated with high-performance liquid chromatography (HPLC)-grade methanol (Merck) and rinsed with acidified Milli-Q water (pH 2). An aliquot of 200 ml of water sample was filtered through a precombusted (450°C) GF/F glass fiber filter and then passed by gravity through the cartridge, which was subsequently extensively rinsed with acidified Milli-Q water (pH 2) and completely dried before elution with HPLC-grade methanol. Each cartridge was eluted with 3 ml of HPLC-grade methanol, and this DOM extract was adjusted to yield almost the same DOC concentration for each sample before injecting to the FT-ICR MS for analysis.

To investigate which fractions of naturally occurring organic matter were oxidized by the COD method, the initial and long-term incubations of river water samples were subjected to COD_{Mn} before DOM extraction. Two milliliters of NaOH solution (250 g liter⁻¹) and 20 ml of KMnO₄ solution (0.01 M) were added to a 200-ml aliquot

of the water sample in a 500-ml conical flask. The mixture was then heated to boil for 10 min. After cooling down to room temperature and being neutralized by HCl (HPLC grade) to pH 7, 0.02 g of NaHSO₃ (Sigma-Aldrich) was added to the mixture to reduce the residue KMnO₄. Then, DOM was extracted using the standard solid-phase extraction procedure described above. As a control, 200 ml of Milli-Q water was used for the COD treatment described above, and DOM molecules commonly existing in the control and the COD_{Mn}-treated water samples were excluded from further analyses. All the glassware used for COD treatments was acid-cleaned and combusted (480°C for 4 hours).

The DOM extracts were adjusted to yield approximately 25 mM DOC and analyzed using a Bruker Apex Ultra FT-ICR mass spectrometer equipped with a 9.4-T superconducting magnet. Sample solutions were infused via an Apollo II electrospray ion source (ESI) at 180 μ l hour⁻¹ with a syringe pump. Typical operating conditions for negative ESI were as follows: spray shield voltage, 3.5 kV; capillary column initial voltage, 4 kV; and capillary column end voltage, -320 V. The mass range was set to m/z 150 to 800. The 4M word size was selected for the time domain signal acquisition. A number of 128-time domain signals were co-added to enhance the signal-to-noise ratio and dynamic range. The magnitude threshold for the peak assignment was set to a signal-to-noise ratio of ≥ 4 . The FT-ICR MS was calibrated using a known homologous series of the Suwannee River natural organic matter sample (obtained from the International Humic Substances Society, USA), which contained a relatively high abundance of oxygen-containing compounds. Molecular formulae were assigned with the calibrated mass data using in-house software (40). The elemental compositions were assigned from the m/z peaks using a mass calculator program limited to molecular formulae consisting of ¹²C₀₋₁₀₀, ¹³C₀₋₂, ¹H₀₋₂₀₀, ¹⁴N₀₋₅, ¹⁶O₀₋₃₅, ³²S₀₋₂, and ³⁴S₀₋₁ (40, 42). All assigned formulae had to meet the following basic chemical criteria: (i) the number of H atoms should not exceed 2C + N + 2; (ii) the sum of H and N atoms should be even (the “nitrogen rule”); and (iii) the number of N or O atoms should not exceed the number of C atoms (43, 44).

Proposed new technique for BOD determination:

The oxygen-optode BOD method

Oxygen optodes are optical sensors that are based on a chemical indicator (dynamic fluorescence quencher) (45). These robust and high-resolution sensors (46, 47) have been used to study a number of oxygen-related processes in freshwater and marine systems (48–52), yielding the same sensitivity as the chemical Winkler method (53, 54). For example, oxygen optodes have already been used in oceanography for measurements of community respiration rates in the mesopelagic and surface waters of the oligotrophic open ocean (48–50). They have also been used for measuring BOD in wastewater (55, 56). Here, we propose a novel, modern BOD method using oxygen optodes instead of the traditional Winkler titration for the measurement of dissolved oxygen concentration [O₂] in the BOD bottles. Oxygen optodes allow the recording of oxygen consumption in the incubated BOD bottles continuously in a noninvasive and nondestructive manner so that the initial and final dissolved oxygen determinations can be conducted in the same BOD bottles (fig. S4). This is a major advantage over the Winkler method, where the initial and final titrations must be performed in different bottles. After proper calibration of the optodes (see below), the [O₂] and thus BOD values meet the quality standard of those obtained with the conventional Winkler titration method.

A detailed description of the BOD optode method will be provided in a separate specialized paper. Briefly, the steps are as follows: (i) water sampling and filling of BOD bottles, which are incubated for 5 days; (ii) oxygen optode measurements in triplicate incubated BOD bottles; and (iii) calculation of BOD₅ (where the index 5 stands for 5 days) from oxygen-optode measurements.

Before using the OXSP5 optodes (PyroScience, Germany) for measuring O₂ in samples, we calibrated them with Winkler titrations. We used a Metrohm autotitrator (888 Titrando with a combined platinum ring electrode) for the Winkler O₂ determinations. The 5-day incubations were conducted at 20 \pm 0.1°C. Winkler O₂ was measured at $t = 0$ every second hour until $t = 8$ hours and every day until day 5 on triplicate bottles during the course of the incubation (total of 30 bottles). The optode signal was measured every 10 min in triplicate bottles during the course of the incubation, with one measurement every 3 s for 2.5 min to acquire 50 values at each Winkler sampling point (total of three bottles).

We tested the validity of the optode BOD method on a variety of natural freshwater and seawater samples. One example was from the DaRen River, Qingdao, China. The results showed good agreement between the optode and Winkler measurements (fig. S5). The coefficient of variation (CV) of the measurements by the optode method (CV = 0.0824; $n = 145$) was an order of magnitude lower than that of the Winkler method (CV = 0.4472; $n = 42$), indicating that the novel oxygen-optode approach can improve the reliability of the oxygen measurement in a BOD determination.

In summary, the novel oxygen-optode BOD method offers several advantages over the traditional Winkler method. First, it provides a more precise measurement of BOD, i.e., replicates from a given sample measured by the optode method reveal a much smaller SD than the respective Winkler replicates do. Second, it eliminates the chemical waste associated with the Winkler method and the need to take into the field chemicals that are toxic to aquatic life such as alkaline iodide azide and MnCl₂. Last, it removes operator error associated with labor-intensive titrations.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/7/16/eabc7318/DC1>

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