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# Improvement of the analysis of the biochemical oxygen demand (BOD) of Mediterranean seawater by seeding control

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ABSTRACT

Biochemical oxygen demand (BOD) is a useful parameter for assessing the biodegradability of dissolved organic matter in water. At the same time, this parameter is used to evaluate the efficiency with which certain processes remove biodegradable natural organic matter (NOM). However, the values of BOD in seawater are very low (around 2 mg $O_2$ $L^{-1}$) and the methods used for its analysis are poorly developed. The increasing attention given to seawater desalination in the Mediterranean environment, and related phenomena such as reverse osmosis membrane biofouling, have stimulated interest in seawater BOD close to the Spanish coast. In this study the BOD analysis protocol was refined by introduction of a new step in which a critical quantity of autochthonous microorganisms, measured as adenosine triphosphate, is added. For the samples analyzed, this improvement allowed us to obtain reliable and replicable BOD measurements, standardized with solutions of glucose–glutamic acid and acetate. After 7 days of analysis duration, more than 80% of ultimate BOD is achieved, which in the case of easily biodegradable compounds represents nearly a 60% of the theoretical oxygen demand. $BOD_7$ obtained from the Mediterranean Sea found to be $2.0 \pm 0.3$ mg $O_2$ $L^{-1}$ but this value decreased with seawater storage time due to the rapid consumption of labile compounds. No significant differences were found between two samples points located on the Spanish coast, since their organic matter content was similar. Finally, the determination of seawater BOD without the use of inoculum may lead to an underestimation of BOD.



## 1. Introduction

Biochemical oxygen demand (BOD) is a widely used parameter for water biodegradability. Direct application allows analysis of the efficiency of a given water treatment process in the reduction of biodegradable natural organic matter (NOM). This analysis establishes the oxygen required for the biochemical degradation of organic material (carbonaceous demand) in a determinate incubation time $t$, in general 5, 7 or 21 days ($BOD_5$, $BOD_7$ or $BOD_{21}$). $BOD_t$ is measured as the difference between the initial dissolved oxygen concentration ($DO_0$) and the dissolved oxygen concentration after $t$ days ($DO_t$) and it is expressed in mg $O_2$ $L^{-1}$.

The BOD protocol is well defined by national and international standards applicable to freshwater and wastewater [1,2], but is unsatisfactory for saltwater or seawater, in which most dissolved organic matter is resistant to microbial oxidation [3]. Indeed, a small labile fraction of dissolved organic carbon (DOC) supports bacterial metabolism [4]. Up to now, few reliable results of seawater BOD have been found in the literature [5–9]. Most of these studies were used to verify the sensitivity of biosensors and the protocols used are poorly described.

In most BOD analyses, the water to be analyzed contains microorganisms but not enough to ensure that the biodegradable NOM is the limiting component. Therefore, standard BOD protocols establish an initial seeding step. Indeed, seawater does not contain a large enough microbial population to ensure the limiting control of the biodegradable NOM. Commercial lyophilized

*Abbreviations:* $Abs_{254}$, absorbance at 254 nm measured in 10 cm cell path length ($cm^{-1}$); ATP, adenosine trhiphosphate (RLU); BOD, biochemical oxygen demand of a sample (mg $O_2$ $L^{-1}$); $BOD_5$, biochemical oxygen demand of a sample after 5 days (mg $O_2$ $L^{-1}$); $BOD_7$, biochemical oxygen demand of a sample after 7 days (mg $O_2$ $L^{-1}$); $BOD_{21}$, biochemical oxygen demand of a sample after 21 days (mg $O_2$ $L^{-1}$); $BOD_t$, biochemical oxygen demand of a sample after t days (mg $O_2$ $L^{-1}$); $BOD_{t\text{-blank}}$, biochemical oxygen demand of blank sample after t days (mg $O_2$ $L^{-1}$); $BOD_{t\text{-SS}}$, biochemical oxygen demand of standard solution sample after t days (mg $O_2$ $L^{-1}$); $DO_0$, dissolved oxygen of a sample at $t = 0$ days (mg $O_2$ $L^{-1}$); $DO_{0\text{-blank}}$, dissolved oxygen of blank sample after $t = 0$ days (mg $O_2$ $L^{-1}$); $DO_{0\text{-SS}}$, dissolved oxygen of standard solution sample at $t = 0$ days (mg $O_2$ $L^{-1}$); $DO_t$, dissolved oxygen of a sample at $t = t$ days (mg $O_2$ $L^{-1}$); $DO_{t\text{-blank}}$, dissolved oxygen of blank sample after $t = t$ days (mg $O_2$ $L^{-1}$); $DO_{t\text{-SS}}$, dissolved oxygen of standard solution sample at $t = t$ days (mg $O_2$ $L^{-1}$); DOC, dissolved organic carbon (mg $O_2$ $L^{-1}$); GGA, glucose–glutamic acid (mg $O_2$ $L^{-1}$); MW, mass weight (g $mol^{-1}$); NOM, natural organic matter; $OD_{Th}$, theoretical oxygen demand of a substance (mg $O_2$ mg substance$^{-1}$); RLU, relative light units; RSW, raw seawater; UBOD, calculated ultimate biochemical oxygen demand according to Thomas [20,21] (mg $O_2$ $L^{-1}$).

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**Table 1**
Basic parameters of RSW analyzed in this work. The seawater source is located at El Prat de Llobregat (Barcelona, Spain) at 2 km from the coast and 25 m below the sea surface.

| Parameter | Value |
|---|---|
| pH | 8.2 |
| TDS (g NaCl/L) | $32.5 \pm 0.6$ |
| Conductivity (mS/cm) | $57.4 \pm 0.4$ |
| Total bacterial count (cells/mL) | $5.2 \times 10^5 \pm 2.0 \times 10^5$ |
| Algae (cells/mL) | $264 \pm 120$ |
| DOC (mg C/L) | $0.79 \pm 0.10$ |
| $Abs_{254}$ ($cm^{-1}$) | $0.006 \pm 0.001$ |

microorganisms from different brands can be used in freshwater or wastewater analysis, but they are not effective in seawater, which has a very different ionic strength [10].

This study aims to demonstrate that a reliable Mediterranean seawater BOD analysis can be performed by establishing an appropriate seeding method.

## 2. Materials and methods

### 2.1. Seawater samples

Raw seawater (RSW) was collected from the Mediterranean Sea at the desalination plant at El Prat de Llobregat (Spain), where the water is pumped from the offshore open intake located 2 km from the coast at a depth of 25 m. Table 1 lists some of the parameters measured in this water.

### 2.2. Cleaning protocol

All glass material was carefully cleaned before use on account of the very low organic matter concentration to be measured. First, glass material was soaked in HCl aqueous solution (10 vol.%) for 24 h and then rinsed with large amounts of MilliQ water. After this, the material was covered with aluminium foil and heated to 450 °C for 4 h.

### 2.3. BOD test

The method used for BOD determination is adapted from the Closed Bottle Method as described elsewhere [11]. Thus samples were incubated for a determinate time at $20 \pm 1$ °C (incubator Medilow Selecta, Spain) in 250 mL nominal volume ISO bottles (Schott, German) closed with a GL45 stopper with PTFE seal to ensure air tightness. The oxygen was measured by HQ40d meter integrated with IntelliCAL™ LDO probe (Hach, USA). Each sample was analyzed in triplicate.

### 2.4. Buffer and salts

According to [1,2], a pH-buffer and mineral salts (inorganic nutrient) are added to the sample: 0.1 vol.% of phosphate buffer of pH 7.2–8.2 (8.5 g $L^{-1}$ $KH_2PO_4$, 21.75 g $L^{-1}$ $K_2HPO_4$, 33.4 g $L^{-1}$ $Na_2HPO_4{\cdot}7H_2O$, 1.7 g $L^{-1}$ $NH_4Cl$) and 0.1 vol.% of salt solutions (22.5 g $L^{-1}$ MgSO4·$7H_2O$, 27.5 g $L^{-1}$ $CaCl_2$ anhydrous, 0.25 g $L^{-1}$ $FeCl_3{\cdot}6H_2O$) (Panreac, Spain). To prevent nitrification, 1 wt.% of allylthiourea (ATU) was introduced as chemical inhibitor [12].

### 2.5. Blanks

Blanks were analyzed in order to control the endogenous respiration of the seed. They were prepared with MilliQ water spiked with 32 g $L^{-1}$ of pure NaCl (Panreac, Spain) in order to meet the seawater salinity.

**Table 2**
Theoretical oxygen demand ($OD_{Th}$) for the different standard compounds used in this work.

| Substance | $OD_{Th}$ (mg $O_2$ mg substance$^{-1}$) |
|---|---|
| Glucose ($C_6H_{12}O_6$) | 1.07 |
| Glutamic acid ($C_5H_9NO_4$) | 0.98 |
| Acetate ($C_2H_3O_2^-$) | 0.95 |

### 2.6. Calculations

$BOD_t$ is defined as the difference between the initial dissolved oxygen concentration and the dissolved oxygen concentration after $t$ days. This corresponds to the total oxygen consumed during the incubation time. Nevertheless, a part of this oxygen corresponds to the endogenic respiration (seed respiration) determined by the BOD of the blank that was run in parallel.

$$\text{BOD of the blank}: \quad BOD_{t-\text{blank}} = DO_{0-\text{blank}} - DO_{t-\text{blank}} \tag{1}$$

$$\text{BOD of the sample}: \quad BOD_t = (DO_0 - DO_t) - BOD_{t-\text{blank}} \tag{2}$$

### 2.7. Seed activity

Measurement of adenosine triphosphate (ATP) was used to monitor inoculum activity. Therefore, the ATP content is proposed as an indicator of biomass content, since ATP is a biomolecule present in all viable microorganisms [13,14]. ATP was quantified using the BacTiter-Glo™ Microbial Cell Viability Assay (Promega Biotech Iberica, Spain) and a GloMax® 20/20 luminometer (Promega Biotech Iberica, Spain). For ATP quantification, the ATP reagent and seawater sample were transferred to an Eppendorf tube (100 μL:100 μL). The mixture was shaken at room temperature, for 3 min and then the luminescence signal, measured as relative light units (RLU), was recorded exactly 3 min and 30 s after ATP reagent addition. All the ATP analyses were performed in triplicate. The results were expressed as RLU and so they allowed the quantification of the biomass activity for each set of BOD analysis.

### 2.8. Inoculum

Autochthonous inocula were obtained from a physical separation process: firstly by sedimentation and secondly by centrifugation or cross-flow filtration [15]. Fresh inoculum can be used as well as lyophilized one (stored in a desiccator at room temperature).

20 mL of seed (6.5 vol.%) was used in each BOD determination.

### 2.9. Bioassay standard solution

As described above the BOD test is a bioassay that requires seed activity to be monitored first. Neutral carbohydrates and amino acids are the main biochemical contributors to seawater DOC [16,17]. Eaton and Franson [1] propose the mixture of glucose–glutamic acid (GGA) as a BOD standard to check seed activity. In this study, sodium acetate and GGA were used as BOD standards.

Environmental Protection Agency [11] establishes an equation to calculate the theoretical oxygen demand ($OD_{Th}$) of a substance, of molecular formula $C_CH_HN_NO_O$ and with determined molecular weight (MW):

$$OD_{Th} = \frac{16\,[2C + 1/2(H - 3N) - O]}{MW} \tag{3}$$

This calculation implies that C is mineralized into $CO_2$ and H into $H_2O$ and that N is transformed into $NH_3$. Table 2 shows the $OD_{Th}$ of the standard compounds used in this study.

Freshly made solutions of 100 + 100 mg $L^{-1}$ glucose (Panreac, Spain) and glutamic acid (Sigma-Aldrich, Spain) and 200 mg $L^{-1}$

**Table 3**
Average $BOD_7$ obtained for the stored and fresh RSW from Mediterranean Sea (Spanish Coast).

| Sample | $BOD_7$ ($mg\,O_2\,L^{-1}$) |
|---|---|
| Stored RSW (without seeding) | 0.2 ± 0.0 |
| Stored RSW | 0.8 ± 0.1 |
| Fresh RSW | 2.0 ± 0.3 |

of acetate (Sigma-Aldrich, Spain), with $OD_{Th}$ of 205 $mg\,L^{-1}$ and 190 $mg\,L^{-1}$, respectively, were used to prepare final standard solutions of 4 $mg\,L^{-1}$ of GGA and acetate in MilliQ water with 32 $mg\,L^{-1}$ of NaCl. The $BOD_t$ of these standard solutions ($BOD_{t\text{-}SS}$) was calculated as follows:

$$BOD_{t-SS} = (DO_{0-SS} - DO_{t-SS}) - BOD_{t-blank} \quad (4)$$

### 2.10. DOC analysis

DOC was determined by the high-temperature catalytic oxidation method (TOC-$V_{CSH}$, Shimadzu), after sample filtration through a 0.2 μm syringe filter. To avoid all inorganic forms of carbon, samples were previously acidified to pH 2–3 (50 μL of 2 M $H_3PO_4$ in 10 mL glass vials) according to [18]. Due to the low NOM content, it should be noted that DOC analysis could be affected by an error of ±0.05 $mg\,L^{-1}$.

## 3. Results and discussion

### 3.1. BOD of seawater: seed control

To determine the amount of seed microorganisms required for a reliable BOD determination, a series of experiments were performed with different quantities of seed measured as ATP concentration. The $BOD_7$ results for RSW are shown in Fig. 1. Two types of RSW were tested: (i) fresh RSW analyzed not later than 6 h after sampling and (ii) RSW stored at 4 °C in the dark for more than 1 day but less than 1 month.

Fig. 1 shows that, on increasing seed content, BOD also increases until it reaches a plateau and does not affect $BOD_7$ determinations of either fresh or stored RSW from the Mediterranean Sea. However, $BOD_7$ values of blank varied linearly with the amount of seed due to inoculum respiration (see Fig. 2). This key observation supports the existence of a threshold seed quantity, enabling replicable and reliable values of BOD to be obtained from the Mediterranean Sea close to the Spanish coast.

For a stored RSW, the minimum activity of seed appears to be about $5 \times 10^6$ RLU; and for fresh RSW, $10^7$ RLU. Note that lyophilized seed was used for stored RSW. Of course, $BOD_7$ without seeding underestimated its value, as was also seen in other studies [4].

Table 3 summarizes the average $BOD_7$ obtained in this study for stored and fresh RSW.

Stored RSW has an average value of 0.8 ± 0.1 $mg\,O_2\,L^{-1}$ for $BOD_7$ at both coastal Spanish sites analysed. Moreover only slight variations were observed with time (see Fig. 3). This observation is consistent with the slight differences in BOD found in [5] over a long period of time (April–September).

$BOD_7$ of fresh RSW from El Prat de Llobregat has higher variability of 2.0 ± 0.3 $mg\,O_2\,L^{-1}$ probably due to daily variations. Differences between fresh and stored seawater are consistent with the findings of [19]. Indeed, bacterial activity probably occurs during storage and is accompanied by oxygen and organic matter consumption, even at low temperature. Furthermore, fresh seawater involves highly biodegradable NOM [16]. Thus all the more readily biodegradable NOM in the water that has been stored for more than one day is consumed.

**Table 4**
Seawater $BOD_5$, $BOD_7$ and $BOD_{20}$ from different sources.

| Sampling point | $BOD_5$ ($mg\,O_2\,L^{-1}$) | Reference |
|---|---|---|
| Dapeng bay (Shenzhen sea area, China) E 114°27′44″–E 114°28′57″ N 22°29′44″–N 22°31′32″ | April; 1.7 ± 0.4 ($n$ = 6)<br>July; 1.6 ± 0.4 ($n$ = 6)<br>September; 1.7 ± 0.5 ($n$ = 6) | [5] |
| Dalian (China) E 121°44′/N 39°01′ | 1.8 ± 0.7 ($n$ = 6) | [6] |
| Xiamen (China) Xiamen University area | Tide; 2.1 ± 0.3 ($n$ = 3)<br>Refluent; 2.4 ± 0.5 ($n$ = 3) | [7] |
| Xiamen (China) Living area around Xiamen University | Tide; 4.1 ± 0.4 ($n$ = 2)<br>Refluent; 6.0 ± 0.5 ($n$ = 2) | [8] |
| Xiamen (China) Around Xiamen University | Living area – Tide; 3.3 ± 0.4 ($n$ = 8)<br>Living area – Refluent; 4.1 ± 0.8 ($n$ = 8)<br>Swimming site – Tide; 2.1 ± 0.2 ($n$ = 3)<br>Swimming site – Refluent; 3.2 ± 0.2 ($n$ = 3) | [9] |
| Santa Rosa Sound (USA) | April; 2.5[a]\|1.5[b]<br>February; 3.3[a]\|2.7[b]<br>January; 1.7[a]\|1.7[b] | [4] |

[a] Corresponding to $BOD_{20}$.
[b] $BOD_7$ from graphic interpolation.

Table 4 compares literature values of $BOD_5$, $BOD_7$ and $BOD_{20}$ of seawater from various sources (mainly from China). The majority of BOD values given in Table 4 are consistent with those obtained by us in this study for Mediterranean fresh seawater. Note that sampling near an inhabited area involves higher $BOD_5$ values, surely due to nearby wastewater disposals.

### 3.2. BOD of standard solution & kinetics

To assess the effectiveness of the seeding and to test the confidence of the method, GGA and acetate standards were added to salt water (32 $g\,L^{-1}$ of NaCl) at a final concentration of 4 $mg\,L^{-1}$. Fig. 4 displays the variation with time incubation of the $BOD_t/OD_{Th}$ ratio for RSW and for GGA and acetate solutions (4 $mg\,L^{-1}$) in salt water.

Results show that, after 7 days incubation, 56% of $OD_{Th}$ for GGA and 67% for sodium acetate were achieved (see Fig. 3). This proves that simple biodegradable compounds are easily biodegraded by the bacterial population [4]. It should be noted that, after 7 days incubation, the reaction kinetics decrease greatly and the BOD reaches a plateau, probably due to the lack of labile compounds, even when there is still enough dissolved oxygen [19].

The use of $BOD_7$ instead of $BOD_5$ is justified because, after 5 days running, the kinetics was still in the final part of linear growth.

Table 5 gives the determined kinetic constants, the resulting extrapolated ultimate BOD (UBOD) and the $BOD_7/OD_{Th}$ and $BOD_7$/UBOD ratios for each sample. Note that $BOD_7$ was calculated according to Thomas [20,21]. Therefore, no significant differences were obtained between GGA and acetate, in which the degree of oxidation ($BOD_7$/UBOD) was in both cases over 70% of UBOD. Consequently, amino acids (like glutamic acid) and other carbon sources (like glucose and acetate solutions) are equally consumed after enough incubation time, suggesting that nitrogen is not a limiting element due to the addition of enough N-salts and only the carbonaceous oxygen demand is measured. Notwithstanding, the rates at which different compounds reach the BOD plateau are different. Acetate and RSW from el Prat de Llobregat have a higher kinetic constant ($k$ = 0.23 $d^{-1}$) than GGA ($k$ = 0.19 $d^{-1}$).

To validate the results obtained and to confirm analysis reliability, [1] proposes to use for a stock solution of 300 $mg\,GGA\,L^{-1}$, and

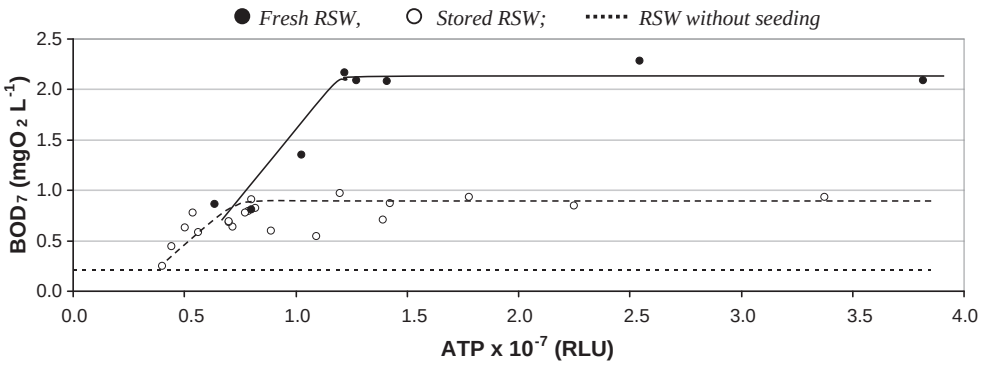


**Fig. 1.** Evolution of $BOD_7$ of RSW (El Prat de Llobregat, Spain) with the amount of seed measured as ATP (RLU): (●) Fresh RSW, (○) Stored RSW; (- - -) RSW without seeding.

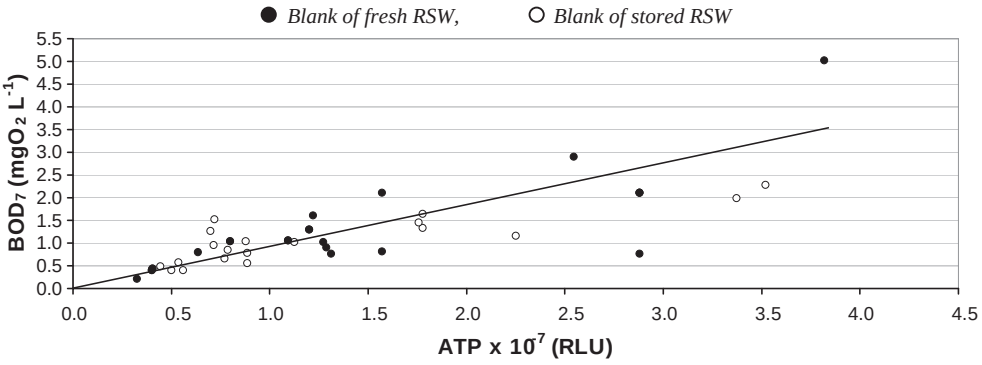


**Fig. 2.** Evolution of $BOD_7$ of RSW (El Prat de Llobregat, Spain) blanks with the amount of seed measured as ATP (RLU): (●) Blank of fresh RSW, (○) Blank of stored RSW.

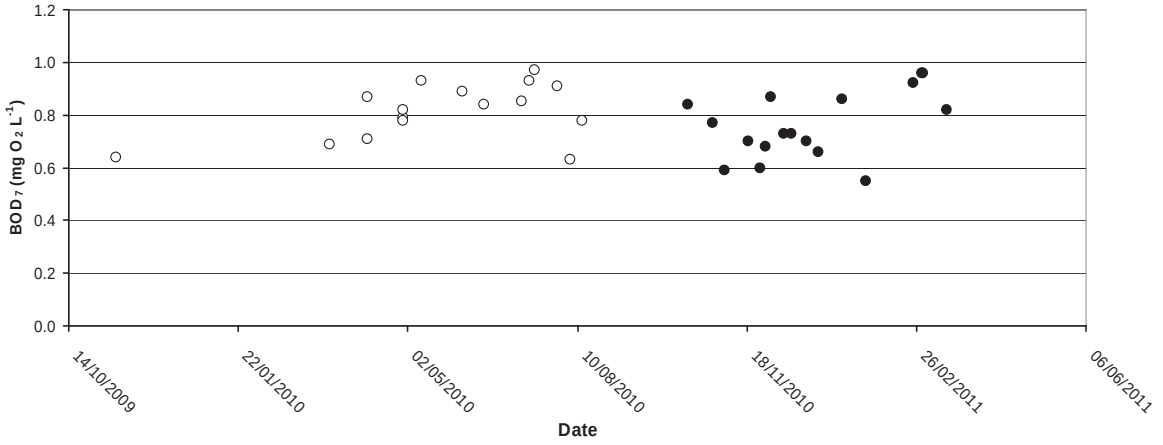


**Fig. 3.** Evolution of stored Mediterranean Sea $BOD_7$ by date of two Spanish coast sample points. (○) Desalination Plant (El Prat de Llobregat)|2 km from the coast and 25 m depth. (●) Mediterranean Center for Marine and Environmental Research (Barcelona)|300 m from the coast and 10 m depth.

after 5 days of incubation, nearly 2/3 of mineralization corresponding to $198 \pm 30.5$ mg $O_2$ $L^{-1}$. The kinetic constant then corresponds to about 0.21 $d^{-1}$ meaning $BOD_7/OD_{Th}$ range between 56% and 67%. Therefore, the ratio of mineralization ($BOD_7/OD_{Th}$) obtained for both GGA and acetate solution is in accordance with [1] and thus confirms the right biomass activity.

### 3.3. DOC and BOD of seawater, GGA and acetate over time

The variation over time of DOC, during incubation, confirms that the consumption of GGA and acetate after 7 days is almost complete. 92% of initial DOC was consumed in both cases (see Fig. 5). In contrast, even the RSW studied gave respiration that

**Table 5**
Kinetic constant ($k$), $BOD_7$, UBOD and the ratios $BOD_7/OD_{Th}$ and $BOD_7$/UBOD.

| Sample | $k$ ($d^{-1}$) | $BOD_7$ (mg $O_2$ $L^{-1}$) | UBOD (mg $O_2$ $L^{-1}$) | $BOD_7/OD_{Th}$ (%) | $BOD_7$/UBOD (%) |
|---|---|---|---|---|---|
| Stored RSW[a] | 0.23 | 0.8 | 1.0 | – | 82 |
| GGA | 0.19 | 2.3 | 3.1 | 56 | 74 |
| Acetate | 0.23 | 2.6 | 3.2 | 67 | 80 |

[a] RSW from Mediterranean Sea (Spanish coast).

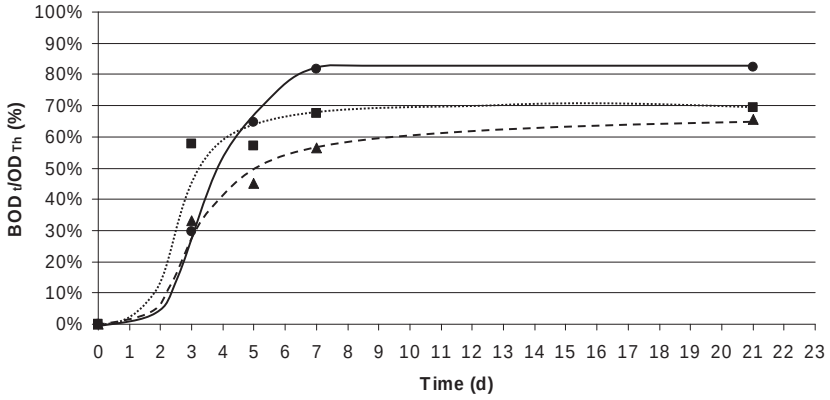


**Fig. 4.** Time evolution of $BOD_t/OD_{Th}$ for RSW (El Prat de Llobregat, Spain) and salt water with 4 mg $L^{-1}$ GGA and 4 mg $L^{-1}$ acetate (● RSW, ▲ GGA, ■ Acetate).

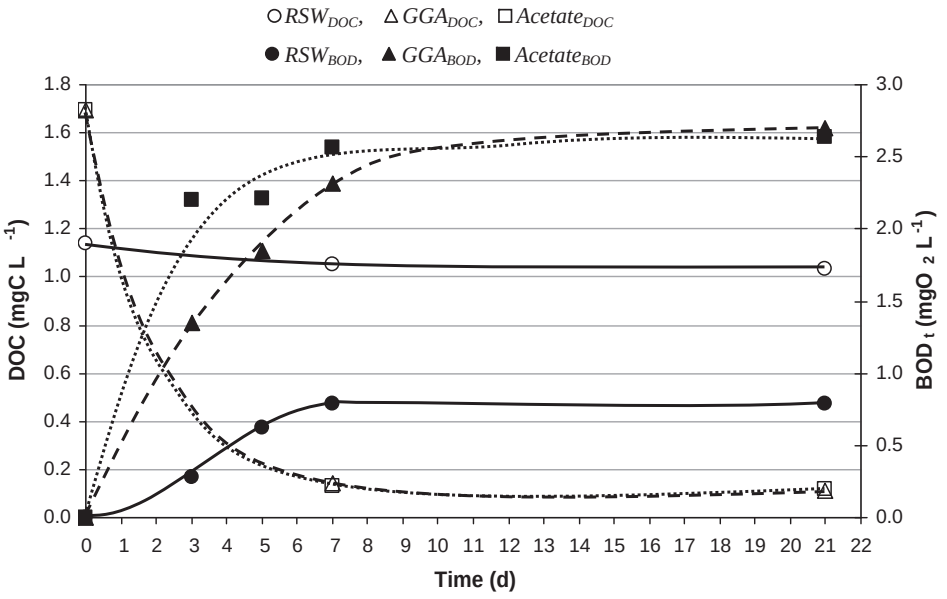


**Fig. 5.** Time evolution of DOC and BOD during incubation for RSW (El Prat de Llobregat, Spain), 4 mg $L^{-1}$ GGA and 4 mg $L^{-1}$ acetate in salt water: (○) $RSW_{DOC}$, (△) $GGA_{DOC}$, (□) $Acetate_{DOC}$. (●) $RSW_{BOD}$, (▲) $GGA_{BOD}$, (■) $Acetate_{BOD}$.

is one-third of the $BOD_7$ from GGA or acetate solutions; in this case, the mineralization was very low. In fact, after 7 days only 8% of the initial DOC was mineralized. These results could be explained in terms of the biochemical activity of the different compounds. In the BOD test, most labile compounds from water are almost totally consumed [3], while new dissolved organic compounds released due to bacterial activity are more refractory to bacterial degradation [22–26]. Amino acids and neutral sugars are directly taken up by microorganisms [16,27–30]. However, only a small fraction of the DOC pool (1–3%) supports bacterial growth in seawater [4].

## 4. Conclusions

The procedure for BOD determination in Mediterranean seawater requires autochthonous seeding. Results obtained for different quantities of seeding, controlled and quantified by ATP measurements; show that a minimum quantity of inoculum is necessary for reliable determination of the BOD. For the Mediterranean seawater analysed and for other seawater with similar NOM content, this quantity represents a minimum of $5 \times 10^6$ RLU for stored seawater and $10^7$ RLU for fresh seawater. Indeed, good biomass activity is confirmed by known standard BOD additions of GGA and acetate. As a result, $BOD_7$ of $0.8 \pm 0.1$ mg $O_2$ $L^{-1}$ is obtained for stored and $2.0 \pm 0.3$ mg $O_2$ $L^{-1}$ for it fresh. Moreover, note that differences between stored and fresh seawater are due to highly biodegradable NOM, which is consumed in a few hours. Around the Spanish coast, fresh seawater has 60% more $BOD_7$ than stored seawater. In addition, greater deviation is found in fresh seawater samples.

First, after 7 days of incubation, TOC analyses revealed almost total consumption of the GGA and acetate added to water spiked with NaCl. In contrast, when TOC analyses were performed on real seawater from the Mediterranean Sea the oxidation of labile NOM represented only 8%, due to the high presence of recalcitrant NOM. However the BOD kinetics of both GGA and acetate solutions and seawater increased rapidly during the first days of analysis and after 7 days the kinetics decreased because of the lack of biodegradable NOM.

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