Determination of Biochemical Oxygen Demand (BOD)

Preface

The determination of biochemical oxygen demand is always a topic of discussion with regard to the method and its applicability. The respirometric method can be compared to the dilution method, and standard methods are discussed in contrast to self-monitoring methods.
We begin with a brief look at standard texts—both national and international texts as well as those from yesterday and today.

The *DIN 38409 H 51* standard described the determination of the biochemical oxygen demand in n days according to the dilution principle! But did the *DIN 38409 H 52* standard describe the respirometric BOD method? It did not. Part 52 described the determination of oxygen depletion. Nevertheless, the evaluation of oxygen depletion as the BOD was an acceptable interpretation, as shown by the following excerpt from *DIN 38409 H 52*.

(not authorized translation)
"...As can be verified in individual cases, the conditions of certain water samples are such that the oxygen consumption is limited only by the degradable organic substances that are present; in cases where the incubation temperature is $(20\pm1)°C$, the oxygen depletion can be interpreted as the biochemical oxygen demand (BOD_n)...."

According to *DIN 38409 H 52*, the oxygen depletion was determined using a standard method and the value was interpreted as the BOD. Regarding the BOD, this measurement was equivalent to a self-monitoring measurement. For most sewage plant laboratories, this is the method of choice due to the self-monitoring regulations in the respective federal states of Germany.

A glance to the United States shows that the *Standard Methods* include the dilution method as the *5210 B 5-Day BOD Test* and the respirometric method as the *5210 D Respirometric Method (PROPOSED).* The respirometric BOD is an independent method and is no longer quoted via the oxygen depletion, although it is a proposed method. Many questions that come up in practice are described and explained in detail, including the frequently asked question about the comparability of the two methods.

"...The point of common dilution and respirometric BOD seems to occur at about 2 to 3 days incubation for municipal wastewaters..."

The situation in Germany is now similar. The *DIN 38409 H 51* has been replaced by the *DIN EN 1899-1* Euronorm that corresponds to the international ISO 5815. It again refers to the dilution BOD, which now has a somewhat different sample preparation and calculation. The European Norm, *EN 1899-2,* also has the status of a German standard and replaces *DIN 38409 H 52.* It describes the procedure for undiluted samples whose BOD must, however, lie at a low level of between 0.5 and 6 mg/L. The oxygen concentration is determined using a sensor or iodometric titration.
And the respirometric BOD? It was adopted in the 46th installment of the "Deutsche Einheitsverfahren" as the blueprint *H55*—in the form of a proposal similar to that in the USA. It is an independent method and the BOD is no longer indirectly determined by way of the oxygen depletion.

The respirometric method for determining the BOD is the classic self-monitoring method for BOD determination. In addition, a new method has now been developed that is based on the photometric principle. This method is also a self-monitoring method and is particularly advantageous for users who have only a few determinations to make and already own a photometer.

The methods for determining the BOD are described below.

Biological background of all BOD_n determinations

The primary difference between BOD_n determination and other measurements such as pH, conductivity, oxygen, COD, nitrate, etc., is the fact that biological systems rather than chemical or physical characteristics are being examined. The biochemical oxygen demand results from the respiratory processes of microorganisms and

microorganisms are alive!

Let us consider an extreme comparison: Bacteria require certain living conditions just as humans beings do. While we do not feel comfortable at the North Pole or when our food is withheld, bacteria similarly place specific demands on their environment. These can be quite variable and extreme because microorganisms are highly adaptable.

The bacteria that can be expected to be present in a municipal water treatment plant require a pH range around the neutral point of pH 7 and a balanced supply of nutrients that is guaranteed by an adequate pollutant load including carbon, nitrogen and phosphorus. They react to temperature fluctuations with a reduced degradation.

In light of this background, it is easy to understand why operators of biological water treatment plants make an effort to protect their "biology" from harmful foreign influences.

However, these relationships not only affect the water treatment plant directly, but also have an influence on the BOD_n determination.

A BOD_n determination is only possible with an adapted biology that must not be damaged, inhibited or destroyed by the sample.

It is essential that the microorganisms are compatible with the sampled water. For this reason, it is best to use microorganisms that "know" the sampled water, i.e. that have adapted to it.

Waters, or test substances, that contain inhibiting, disinfecting or even toxic agents interfere with the microbiology.
Waters containing these substances do not have a BOD_n.

Measurement results in these waters can only provide information on the toxicity of the substances used.

# Respirometric BOD_n determination

The determination of the BOD concerns the determination of the degradation of organic substances by microorganisms.

The main application of respirometric BOD_n determination lies in the analysis of wastewater in wastewater treatment plants. Respirometric measurement in a bottle corresponds to the processes in a wastewater treatment plant, but on a greatly reduced scale. At the same time, the analysis can be used for various aqueous media, e.g. in flowing or standing surface waters and in natural as well as artificial waters.

The measurement period can vary widely. For the classification and evaluation of the degradation performance of a wastewater treatment plant (with the exception of some Scandinavian countries), it is customary to specify the BOD_5. In this case, the analysis time is 5 days. During this time, the measurement solution must be incubated at 20°C, i.e. the sample bottle is thermostatted to (20 ± 1)°C in an incubator for the entire measurement duration.

Some Scandinavian countries specify the BOD_7 value. In a seven-day incubation period, a measurement that is started on Tuesday is also completed on Tuesday; however, in a BOD_5 measurement, it is completed on Sunday. And who wants to go into the wastewater treatment plant on a Sunday to read the measured values! In the days of the mercury manometer, the BOD_7 measurement offered a distinct advantage as measurements could be made on practically any day. The use of the OxiTop® system now also makes this possible for the BOD_5 measurement because it automatically stores the measured values. The measured values, even those recorded on Sundays and holidays, can be read just as well several days later. A further advantage of the OxiTop® system is, moreover, the mercury-free pressure measurement. Many laws and regulations namely call for the avoidance of chemicals and substances that are injurious to health!

Basics
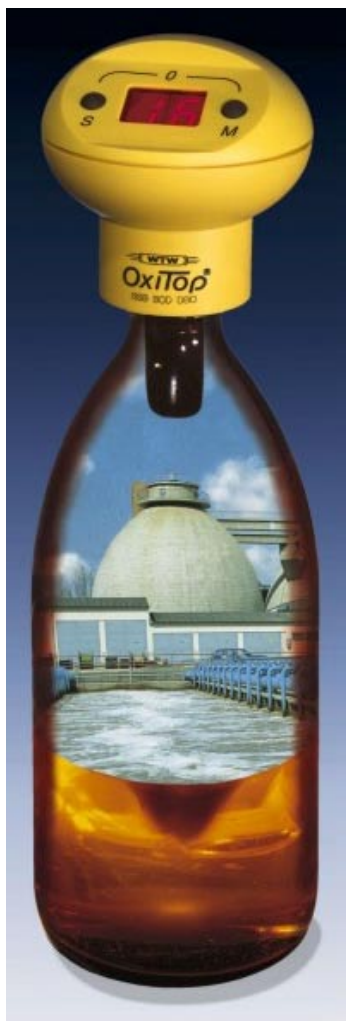
Compared to the other recognized methods, this procedure comes closest to representing the natural conditions of biological degradation.
Interference with the sample solution is kept to a minimum.

Basically, respirometric measurement using the OxiTop® system is nothing more than a small-scale wastewater treatment plant, poured into a bottle and operated in the absence of air.

All of the oxygen required for consumption comes from the graduated measuring flask. This includes not only the dissolved oxygen but also oxygen from the gas phase (air above the measurement solution). The partial oxygen pressures, i.e. the amounts of oxygen in the aqueous phase and in the gaseous phase, are balanced. Constant vigorous stirring ensures a good exchange of gas between the two phases.
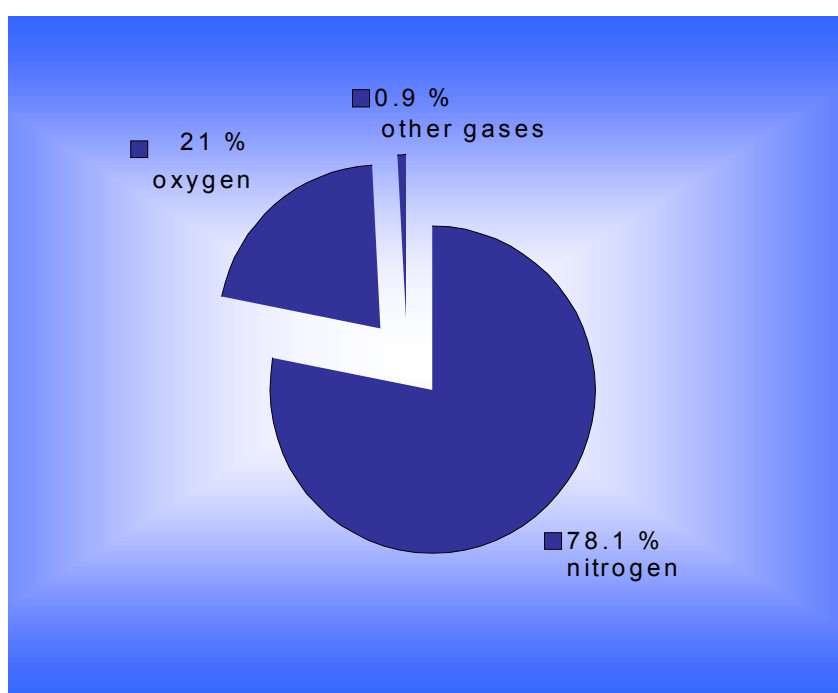
Air is composed of:
- 78.1 % nitrogen,
- 21 % oxygen,
- 0.9% carbon dioxide and noble gases.

Any one of these gases, e.g. oxygen, contributes to the total air pressure in exactly these proportions.

1013 hPa x 0.21 = 213 hPa

That is to say, at an air pressure of 1013 hPa, the partial oxygen pressure is 213 hPa.

Measuring principle

In the same way as we human beings require oxygen, many microorganisms also require oxygen to obtain energy. This biochemical oxygen demand can be determined by measuring this phenomenon. Bacteria inhale oxygen and exhale carbon dioxide.

$$\textbf{C + O}_2$$

$$\textbf{CO}_2\uparrow$$

It is now possible to determine the BOD for the measurement either directly by measuring oxygen or indirectly by measuring carbon dioxide as a molecule of oxygen is converted into a molecule of carbon dioxide. Respirometric methods use carbon dioxide and measure the change in pressure. But where does this change in pressure come from? A mol of oxygen, i.e. $6.022 \cdot 10^{23}$ molecules, has a volume of 22.4 liters. A mol of carbon dioxide, also with $6.022 \cdot 10^{23}$ molecules, has a volume of 22.4 liters, too. If the oxygen is now converted to carbon dioxide by respiration, there is no direct change in pressure. At this point, the role of the sodium hydroxide in the neck of the bottle comes into play. Sodium hydroxide and carbon dioxide react chemically to form sodium carbonate.

$$\textbf{2 NaOH + CO}_2\uparrow \;\rightarrow\; \textbf{Na}_2\textbf{CO}_3\downarrow \textbf{+ H}_2\textbf{O}$$
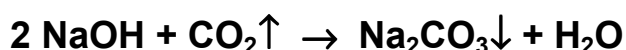
This causes the carbon dioxide that was formed to be removed from the gas phase and results in a measurable negative pressure due to the respiration of oxygen.

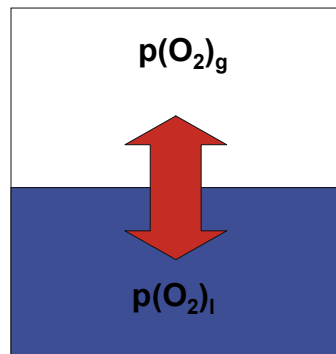**The respirometric measurement is a pressure measurement!**

The measured negative pressure is converted into the BOD value using the following equation.

$$BOD = \frac{M(O_2)}{R \cdot T_m} \cdot \left(\frac{V_{tot} - V_l}{V_l} + \frac{T_m}{T_0} \right) \cdot p(O_2)$$

$M(O_2)$	Molecular weight of oxygen (32000mg/mol)
R	Gas constant (83,144 L·hPa/(mol·K))
T_0	Temperature (273.15 K)
T_m	Measuring temperature (293.15 K) for BOD_5
V_{tot}	Bottle volume [mL]
V_l	Sample volume [mL]
α	Bunsen absorption coefficient (0.03103)
$\Delta p(O_2)$	Difference of the partial oxygen pressure [hPa]

For completeness, it must be added that the equation was derived from the ideal gas law under the conditions of an additional liquid phase.

If the microorganisms consume oxygen in the aqueous phase, oxygen from the gas phase is added as the partial pressures of the gases present constantly adapt.

The partial oxygen pressure is of significance to the respirometric measurement. The partial oxygen pressure in the aqueous phase is the same as the partial oxygen pressure in the gas phase.

$$p(O_2)_{fl} = p(O_2)_g$$

In order to accelerate this exchange and to prevent oxygen deficiency in the measurement sample, the material under test is thoroughly mixed during the entire duration of the measurement.

# Brief instructions on how to perform a measurement using the OxiTop® system

1. Estimate the measuring range of the sample to be analyzed.

2. Before filling the overflow measuring flask, add all the additional solutions

3. If required, add the nitrification inhibitor.

4. If necessary, seed the sample (caution: blank test determination!).

5. If necessary, add nutrient solutions, mineral solutions and buffer solutions (caution: blank test determination!).

6. Take the selected volume of homogenized sample with the aid of the overflow measuring flask.

7. By means of a funnel, transfer the measurement solution into the graduated measuring flask.

8. Insert a magnetic stirrer bar into the bottle.

9. Place 2 sodium hydroxide pellets in the rubber sleeve.

10. Insert the rubber sleeve onto the bottle. (Samples that come into contact with sodium hydroxide can no longer be used for measurement.)

11. Screw on the OxiTop® measuring head tightly. The rubber sleeve ensures the necessary sealing of the system. (Do not use any sealing lubricant!)

12. Start the measurement on the OxiTop® head, on the controller, if the OxiTop® C is used.

13. Place the graduated measuring flask in the incubator for five days at 20°C.

14. Read the results after five days

# Components of the respirometric measuring system

Graduated overflow flask
This simplifies the process of obtaining the mostly odd volumes required for the measurement. The expected range of measurement of the sample determines the volume to be used. The two volumes that are most often required are 164mL and 432mL. The volumes used are selected so that the factors for calculating the BOD_5 are even-numbered.
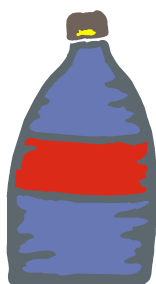
Graduated measuring flask
This is a brown glass bottle that has a capacity of 510 mL and a threaded neck. Brown glass prevents any possible growth of algae. In order to close the graduated measuring flask so that it is leakproof, it is sufficient to tightly screw on the OxiTop® measuring head.
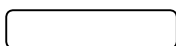
Magnetic stirrer bars
The magnetic stirrer bars that are supplied are designed specially for the bottles so that they provide optimum mixing of the sample. Smaller or larger stirrer bars, or even other shapes, do not necessarily ensure that the sample is completely mixed.

Nitrification inhibitor
The so-called nitrificants (typically nitrosomonas and nitrobacter bacteria) also consume oxygen in the conversion of ammonium to nitrite and then to nitrate. This consumption is not included in the BOD_n value. Consequently, an inhibitor is added to the measurement solution to prevent the conversion of ammonium to nitrate.

Rubber sleeve
The rubber sleeve fulfills two functions: it provides the leakproof sealing of the bottle when screwing on the OxiTop® measuring head and it accommodates the carbon dioxide absorber (sodium hydroxide pellets). The rubber sleeve must not be lubricated. Certain sealing lubricants even destroy the plastic of the measuring head.

Sodium hydroxide pellets
Sodium hydroxide pellets are used to absorb carbon dioxide. 1-3 pellets NaOH are required for each measurement. As a result of the reaction with carbon dioxide in which water is formed and due to the hygroscopic (water-attracting) properties of NaOH, the pellets become damp or are dissolved during the measurement.

OxiTop® measuring heads

OxiTop® measuring head for manual operation of the BOD$_5$ measurement

OxiTop® Control measuring head for the detailed observation of the oxygen degradation curve

5 measured values over 5 days

x measured values over y days

Stirring platform

The stirring platform is available in two sizes: with six or twelve integrated stirring slots in which separate alternating electromagnetic fields are generated. Thus, torn or slipping rubber driving belts are a thing of the past with the use of the inductive stirring system. In addition, magnetic stirrer bars that are out of position or "stuck" are pulled back into the middle of the bottle. Faulty measurements due to a lack of oxygen exchange between the aqueous phase and gas phase are impossible.

The stirring platforms can be operated in a suitable thermostat box, in a normal thermostat cabinet or in a thermostatic room.

Thermostat boxes and cabinets

ensure the necessary temperature regulation to 20.0 ± 0.5 °C. The power supply for the stirring platforms is integrated in the thermostats.
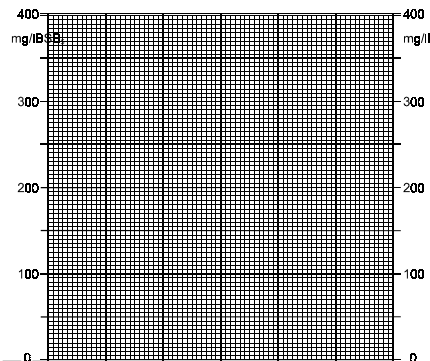
Thermostat cabinet TS

Thermostat box

	1	2	3	4	5	6
Zeichen od. Farbe	X rot	O grün	□ gelb	— blau	△ braun	+ schwarz
Einfüllvolumen						
Faktor						
Entnahmestelle						
Bemerkung zur Probe						

BSB₅ Meßbereich	Einfüllvolumen	Multiplikationsfaktor (F)
0 - 40	432 ml	1
0 - 80	365 ml	2
0 - 200	250 ml	5
0 - 400	184 ml	10
0 - 800	97 ml	20
0 - 2000	43,5 ml	50
0 - 4000	22,7 ml	100

Evaluation sheet
The individual readings of a manual measurement are entered on this sheet and evaluated. (The five values for the five days of the BOD_5 determination are absolutely clear and sufficient.)

Magnetic stirrer bar remover
This is a coated rod with a magnet integrated in its end. After the measurement has been completed, the magnetic stirrer bar can be easily removed with the aid of this magnet.
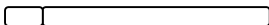
Marking rings
The marking rings are used for better correlation of the graduated measuring flasks to the sample. They are marked with numerals and are pulled over the neck of the bottle before the measuring head is screwed on. (The OxiTop® Control does not require the use of marking rings as it provides automatic sample management.)

Which points must be observed in every BOD_n measurement?

Sampling

If analysis begins within 2 hours of sample collection, the sample does not need to be cooled. Otherwise, the sample must be cooled to <4°C immediately after it has been taken.

The time to analysis must not exceed 6 hours. If this is not possible, the duration and temperature of storage must be noted. The sample must not be stored for more than 24 hours.
The duration of bulk sampling is restricted to 24 hours.
During the sampling of a bulk sample, the sample must be cooled to <4°C. A bulk sample is stored in the same way as a random sample.

A sample is taken using a clean dry vessel and poured into a clean and dry vessel. The sampling vessel is not prerinsed with the sample solution. The sampling volume is at least one liter.
If possible, the sample should not be frozen. Deep-frozen samples result in lower measured values (up to 10% lower). The reason for this is again due to the fact that a biological process is being analyzed:
Ice has a larger volume than water (this is the reason that icebergs float!). As a result, the cell walls of deep-frozen cells can burst and, thus, damage the microorganisms. This inevitably causes the BOD value to fall.

Mixing and homogenizing

The sample must be homogenized. The reason for this is obvious if you imagine a sample that has been allowed to settle. Obviously, measuring the sediment would lead to a BOD that was too high whereas measuring the supernatant liquid would lead to a BOD that was too low. The question remains is what kind of homogenization should be used. The use of a blending machine is only recommended if the particles of solid matter are very coarse. The blending process destroys the flakes and the microorganisms could be damaged. Mechanical stirring or a magnetic stirrer with a stirring rod is gentler.

The user must be made aware of the effect of this process if he wants to homogenize samples with the aid of a blending machine at 20000 U/min. This process can drive out readily volatile substances and destroy flakes in the sludge. If this has no evident effect on the result, a blending machine can be used for homogenization.

While taking the volume required for the measurement with, or in, an overflow measuring flask, it should be constantly stirred. The particulate solids must be evenly distributed throughout the sample. At the same time, it is an advantage if during the filling of the overflow flask, the stirring is only strong enough to prevent the solids settling out.
The sample in the BOD bottle must have a composition that is identical to the original sample.
If graduated cylinders are used, there is a danger that particulate solids may settle out during calibration of the sample.
Pipettes should not be used either. Flakes can be sucked in through the narrow tip of the pipette and block the opening causing it to act as a filter. Measuring such a sample would inevitably lead to lower results.
One point that must also be addressed in this context is filtration. Apart from a few exceptions in the sector of waste water lagoons that may be affected by algae growth, no filtration of samples is required.

BOD samples are not filtered as a rule!

By filtering the sample, undissolved components that naturally also have a BOD are removed. The measurement would lead to lower results.

Thermostatting

The sample and any dilution water that is used must be brought to the required temperature $\pm$ 1°C before dilution and before being poured into the graduated measuring flask. Any sample that ever had a temperature of >50°C at any time must be seeded with a sufficient number of bacteria. The temperature during a BOD_n measurement should be held constant $\pm$ 1°C throughout the entire measurement period.

The OxiTop$^\circledR$ has a built-in AutoTemp function. It is sufficient to thermostat the sample to 15 – 21°C before taking the measurement sample. This point will be addressed separately at a later stage.

Oxygen concentration

The initial concentration of oxygen in the measurement solution is adjusted immediately before the start of measurement. This is done best and most simply by shaking the sample or by aeration with clean filtered compressed air. The measurement sample must be saturated with oxygen so that during the measurement, i.e. during the next five days, the oxygen concentration is not the limiting factor. Otherwise, the air for the bacteria expires in the truest sense of the word and their degradation performance falls. If, in the course of the measurement, sufficient oxygen is no longer available, the measurement result must be discarded. The aerobic degradation process that is to be analyzed no longer takes place.

For application in wastewater analysis, oxygen saturation by shaking the sample for approx. 15 minutes has turned out to be practical. This process raises the oxygen concentration very simply and rapidly up to saturation. The bottle in which the sample was taken should not be completely full for this process. This ensures that the sample comes into contact with plenty of air and that it is gently homogenized at the same time. Actually, many wastewater samples are already saturated with oxygen, however, through constant contact with air (in open flumes).
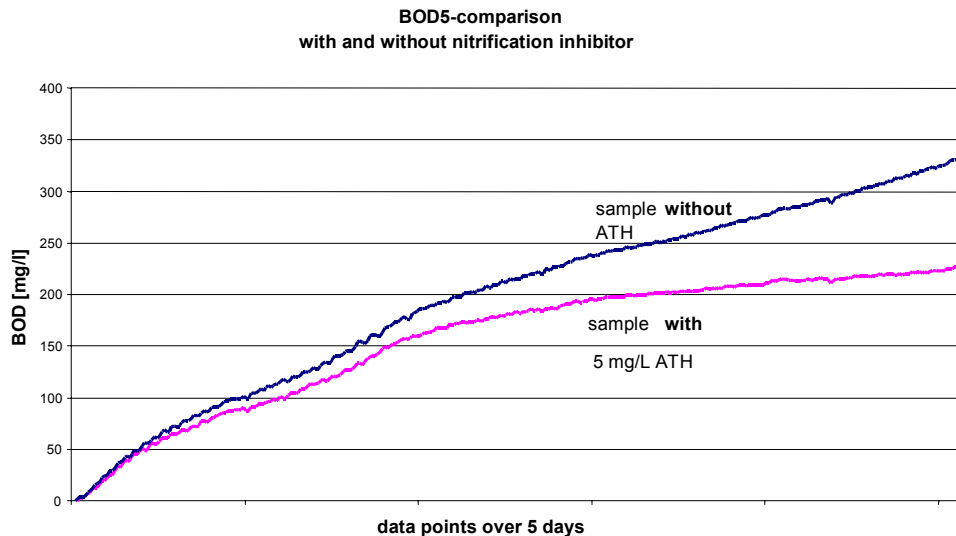
Nitrification inhibitor
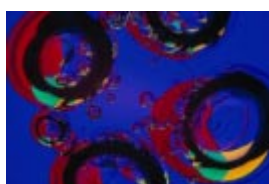
The oxidation of nitrogen from ammonium to nitrate by specific bacteria is called nitrification. This biological process also causes the oxygen to become bound as can be easily seen in the formulas for ammonium (NH_4^+) and nitrate (NO_3^-). The oxygen required for this does not form part of the BOD_n!

This parallel biochemical reaction can be suppressed by the addition of a nitrification inhibitor. The substance normally used for this is allyl thiourea (ATU) or 2-chloro-6-(trichloromethyl)-pyridine (TCMP). The nitrification inhibitor blocks or toxifies the specific bacteria that are responsible for the degradation of the ammonium without, however, damaging the microorganisms that degrade carbon compounds (BOD!).
Up to now, the rule of thumb has been applied where nitrification inhibitor has only ever been used for effluent measurements. However, it is also recommended for influent measurements to safely exclude nitrification by the appropriate additive as it is certainly possible for nitrificants to be present in the wastewater of the wastewater treatment plant influent.

The effect of adding or leaving out the nitrification inhibitor is indicated by the evaluation of a real sample from the effluent of a preclarification shown below.

BOD5-comparison
with and without nitrification inhibitor

The effect of the nitrification inhibitor is shown clearly here. It suppresses the inclusion of the nitrification processes.

Special tip and to recapitulate:

If implausible BOD results occur, we recommend checking the nitrification inhibitor. Implausible results mean BOD values that are too high, e.g. in the order of magnitude of the COD or even higher. The BOD must not, however, exceed the COD as the chemical oxygen demand includes the biochemical oxygen demand. Several points concerning the use of nitrification inhibitor must be observed:
- The concentration of the allyl thiocurea in the sample should be 5 mg/L
- If commercially available ATU solutions are used, ensure that the correct dosage is used. The WTW NTH-600 solution has a concentration of 5 g/L. Accordingly, 20 drops must be added to each liter of sample.
- Even ATU solutions have a best-by date. Solutions that are too old have mostly lost their effect.
- ATU solutions should be stored in the dark (that is why the NTH 600 bottle is made of black, opaque plastic) and, whenever possible, kept cool.

Which points should you observe in a measurement?

Usually, domestic wastewaters can be used for measurements without significant pretreatment. However, the points mentioned in the previous section must always be taken into account! This is not the case in, e.g. industrial wastewater or highly polluted wastewater. To prevent the result from being falsified, a number of possible interference effects depending on the sample must be observed and, if necessary, remedied. For more information, refer to the relevant WTW application reports.

Neutralization
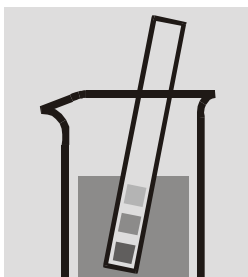
The sample should have a neutral pH value of between 6.6 and 7.2. The pH value can be adjusted by means of sulfuric acid or sodium hydroxide (Appendix R7 and R8).

Microorganisms always adapt to their specific habitat. In order to survive, they require an environment that is suited to their species. Indispensable for this is an adapted pH range within the sample. In the purification of biological wastewater, this corresponds to a pH range of between 6.6 and 7.2.

Inhibiting and toxic components

If a sample contains inhibiting and/or toxic substances such as phenols, heavy metals or cyanide compounds in high concentrations, the samples must be specially monitored and processed.

The oxygen degradation curves in inhibited and/or toxic polluted samples are greatly delayed. In some cases, almost no oxygen degradation can be seen in the first few days whereas, in other cases, degradation is reduced throughout the entire testing period.

Degradation curve for samples with inhibiting and/or toxic components
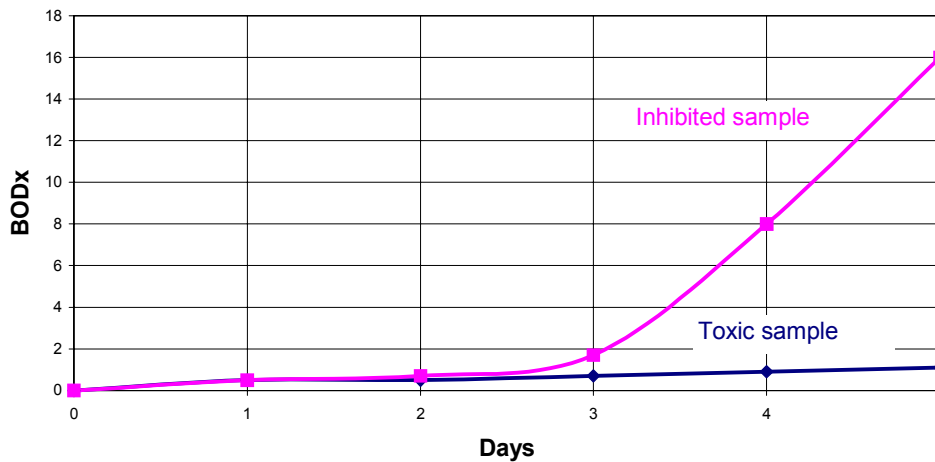
Microorganisms vary in the degree of their reactions to pollutant concentrations. These effects can be reduced or even cancelled by diluting the sample. To do this, various dilutions must be prepared. If two consecutive dilutions result in the same BOD value one after the other, the effect of the toxic substance has been cancelled (do not forget a blank test determination).
Inhibited samples and disinfected samples do not consume oxygen. A BOD_n value is generated as the result of appropriate measures. Furthermore, a statement can be made only on the toxicity and the degradability of the sample.

!   The concentration of inhibiting or toxic substances can be determined using the appropriate photometric test sets.
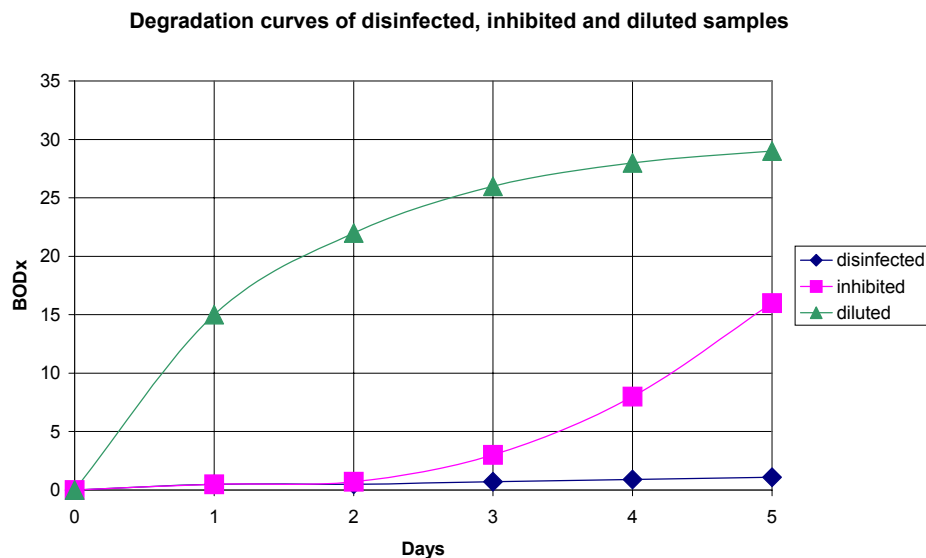
Chlorine or other bacteria-killing substances

Samples that contain chlorine or other bacteria-killing substances should be avoided, e.g. by sampling before any chlorinating process. Chlorine that is present can be removed by blowing clean filtered compressed air through the sample for approximately one hour or by leaving it to stand for 1-2 hours in daylight. If these measures are not sufficient, the chlorine content must be determined, converted to the amount of sample and an adequate amount of sodium sulfite solution added to the sample.

Chlorine or other disinfectant substances are utilized to kill bacteria. This effect is also retained by the wastewater. A BOD_n measurement can then merely provide a statement on the toxicity, e.g. in order to obtain a basis for the decision on the dilution ratios in which the polluted water can be input to the wastewater treatment plant.

Amongst other things, it is possible to determine whether a manufactured disinfectant fulfills its task or how long a substance can last if it is exposed to biological degradation processes. (An additional application of the OxiTop® measurement system are measurements of the biological degradability!) Furthermore, it is possible to cultivate adapted microbiology in this way for precisely outlined problems.

Degradation curves of disinfected, inhibited and diluted samples

Samples that contain chlorine or other bacteria-killing substances have no oxygen consumption. Through dilution and subsequent seeding with bacteria, a BOD_n value can nevertheless still be measured for these samples.

Sample dilution

Dilution of the sample only becomes necessary if, as just touched upon, the concentration of toxic or inhibiting substances needs to be reduced, or if the BOD value of the sample lies above the upper limit of the range of measurement (several 1000 mg/L BOD). The dilution water must be produced under certain conditions that are described below. Essentially, the statutory norms and regulations can also be referred to (e.g. *DIN EN 1899-1*, *DIN 38409 H 51 or Standard Methods 5210 D*) for information on the preparation of dilution water. In any case, the BOD of the dilution water must also be determined (see Application Reports). The BOD_n value to be expected determines the volume of sample that is required. Usually, buffer solutions, nutrients, minerals and nitrification inhibitor are added to the dilution water. Seed that consists of intact and adapted microbiology is also added.
The following data on the reagents that are employed and their concentrations refer to the *Standard Methods 5210 D.* Compositions for special requirements that differ from these are quite conceivable.

Water

Water from different, but suitable, origins can be used for sample dilution, e.g. river water with no organic components, drinking water or distilled water (Appendix A) with additives of various salts and nutrients.
Drinking water must be chlorine-free or rendered chlorine-free by sufficient aeration with compressed air.

Pure distilled water without any electrolyte additives (see dilution BOD) damages the cell as a result of osmotic processes. The inside of the cell has a higher concentration of electrolytes, i.e. dissolved substances. Because distilled water has no electrolyte concentration and this system strives for a concentration balance, water constantly diffuses through the cell wall into the inside of the cell. The cell inflates like a balloon and will burst at some time or other.

Nutrients and buffer solutions

Nutrients such as nitrogen (N) and phosphorus (P) must be available in sufficient amounts. In this case, the C:N:P ratio of 100:5:1 or the TOC($\Rightarrow$ total organic carbon):N:P ratio of 30:5:1 is adhered to. It may, e.g. be necessary to add an appropriate amount of ammonium chloride solution (Appendix R2).

If the C:N:P ratio is disturbed, the under-represented substance has a limiting effect. Even in a wastewater treatment plant, it becomes drastically apparent if the C:N:P ratio is greatly disturbed. This promotes the occurrence of scum or bulking sludge, the age of the sludge changes; the total degradation performance of the plant drops!!!

The phosphorus demand can be covered by phosphate buffer solution (Appendix R1).
1 mL phosphate buffer solution is added per 50 mg/L COD of the diluted sample.

Attention: The toxicity of samples with metal salts can be reduced by phosphate buffer solutions because phosphate complexes can lower the concentration of the metal ions.

Phosphate buffer solution should be used with caution for inhibiting or toxic samples. Since no phosphate buffer solutions are added in big scale wastewater purification, this can lead to incorrect estimations of the actual biological degradability of the water sample.

Minerals and trace elements
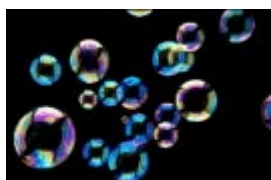
If sufficient amounts of mineral nutrients are not present in the sample solution, 2 mL each of a calcium, magnesium, iron and trace elements solution are added to each liter of dilution (Appendix R3, R4, R5 and R12).

If no minerals and trace elements (or too few) are present in the sample, these must be added. This is because, in exactly the same way as for the nutrients, the under-represented substance hampers the complete degradation of the substances.

Such limitations lead to distortions of the measured values. They simulate the nondegradability of a sample although the degradability could be possible with a sufficient quantity of minerals and trace elements. Here also, a comparison can be

drawn again with human beings in which a lack of trace elements leads to the most varied deficiency illnesses.
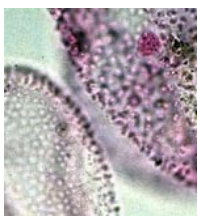
Seed
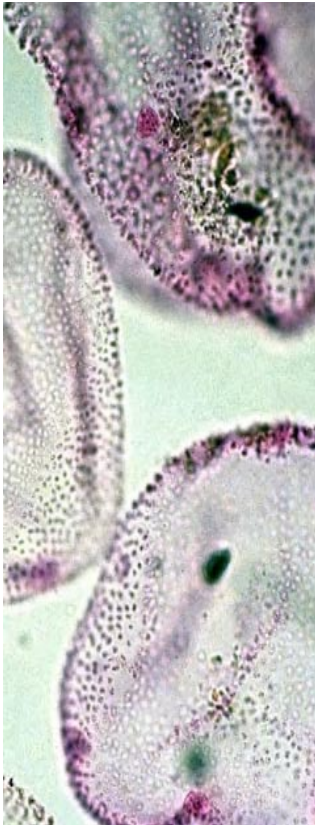
Some samples do not contain the necessary number of microorganisms (for example, industrial wastewater, disinfected water, heated wastewater or waters with extreme pH values and others). Seeding of the samples ensures the presence of sufficient, available microorganisms (Appendix R15). Preferably, adapted seed such as the influent of the biological purification stage of a wastewater treatment plant should be used. Some samples contain components which, under normal conditions, are not degraded by the microorganisms in domestic wastewater. Such samples must be seeded with adapted microbiology. It is also possible to use activated sludge, a commercial seed preparation or microorganisms from soil eluates in order to obtain the required microbiology. If this cannot be obtained, a characteristic adapted seed should be cultivated in which microorganisms are initially brought into contact with the problem substance in a low concentration. Gradually, the concentration of the problem substance is increased. The BOD value is measured over and over again. If the degradation rate with increasing adaption time reaches a stable level, this indicates a successful adaption of the seed.

It has been shown that wastewater from the settled influent to the biological purification stage of a wastewater treatment plant is best suited for the seeding of a test wastewater. Adapted seed is most easily obtained from the wastewater treatment plant that normally purifies the test wastewater.

Domestic wastewater
Samples of domestic wastewater do not normally require seeding with bacteria. They can be used directly as the measurement solution.

Mostly, municipal wastewaters contain sufficient nutrients, minerals and trace elements for the optimum degradation of the carbon compounds. Otherwise, the wastewater treatment plant operator must consider suitable measures such as, e.g. dosing individual substances in sufficient amounts.

Highly organically polluted wastewaters
Samples from the food industry frequently involve highly organically polluted wastewater. In most cases, seeding plays no role here. An adequate supply of nutrients is much more important.

Frequently, the nutrients are the limiting substances in this type of wastewater.

Measurement

Estimating the expected BOD value

In order to select the correct range of measurement for a determination, the BOD_n value is estimated before analysis. The oxygen content in the bottle must not, in any way, become the limiting factor. A BOD measurement is limited by the content of biologically degradable carbons that must be determined! This is countered for a sample with a high BOD value by pouring a low volume of sample into the bottle that has a correspondingly large quantity of oxygen available in the gas phase. For samples with a low BOD, a large amount of sample can be taken to increase the resolution. The oxygen content of the small gas phase is adequate.

The approximate BOD value of the sample must be known in order to estimate the range of measurement.
If no value of experience is available, the following approximation can be used
BOD value = ½ x COD value.
This factor can rise to almost 1 for higher organic pollution.
On the basis of the estimated value, the required volume of sample is selected according to following table.
If the estimated value lies outside the specified ranges of measurement or if a larger volume is to be used, the sample must be diluted.

Expected BOD value [mg/L]	Amount of sample to be used [mL]	Factor (*)
0 – 40	432	1
0 – 80	365	2
0 – 200	250	5
0 – 400	164	10
0 – 800	97	20
0 – 2000	43,5	50
0 – 4000	22,7	100

(*) The OxiTop® Control measuring system does not need to take these factors into account as the result is output directly by the controller in mg/L BOD.
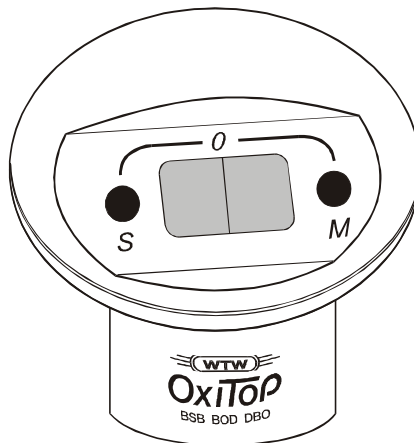
AutoTemp function

The partial steam pressure and the partial pressure of dry air are temperature-dependent.
These complicated facts have a simple and clearly understandable effect as follows:
Samples with a temperature below 20°C expand when they are heated. Consequently, the pressure in a closed bottle with an additional gas phase must increase. Cooling samples that are above 20°C leads to a corresponding volume contraction and, thus, to a negative pressure. As a result, mercury respirometers must remain open for one hour and the sample temperature must be preadjusted to 19-21°C.
The measuring head of the OxiTop® systems with AutoTemp function takes over the control of the temperature adaptation. The OxiTop® with AutoTemp function enables the sample bottles to be closed immediately and the measurement started when the sample temperature is at between 15 and 21°C.
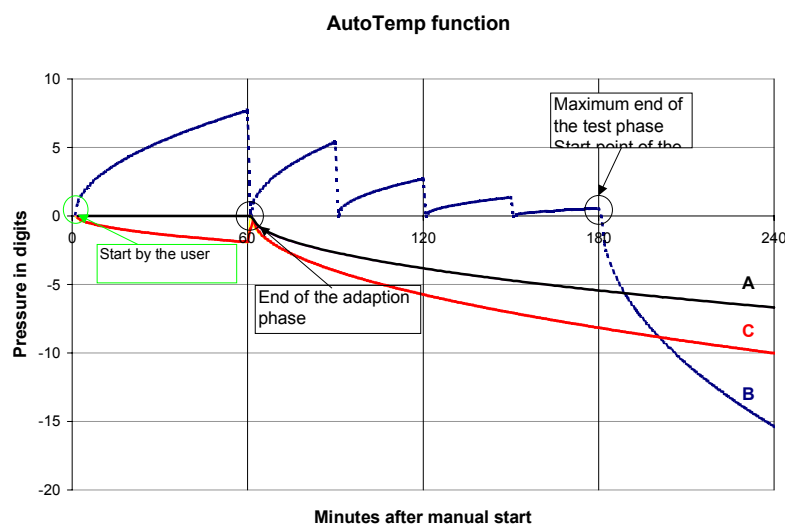
Operation:

The WTW patented AutoTemp function consists of the adaption phase and the test phase. It is automatically activated following the start:
The adaption phase is a phase without evaluation of the pressure (60 min). The microbiology can adapt itself and smaller temperature deviations ±1°C can be balanced out. In addition, the steam saturation equilibrium can be adjusted. After the adaption phase expires, the measuring system is zeroized in any case.
In the subsequent test phase, the remaining temperature deviation can be compensated for a sample with a temperature that is too low.
After a specified time interval that depends on the total measuring duration and is 30 minutes for the BOD_5 measurement, the pressure measurement is repeated. If the pressure has dropped, the sample has a temperature of 20°C and the BOD process can be measured. The OxiTop® begins the measurement and uses this as the initial value. However, if the pressure has increased, the sample was not yet thermostatted (< 20°C) and the system is set back to "zero" again. This is repeated until the pressure measurement registers a constant pressure or a drop in pressure.

Graphically, this appears as follows:

AutoTemp function

Curve A:
The temperature of the sample is adjusted (19-21°C). **Optimum range.**

Curve B:
The adaptation takes a very long time. The measurement solution being used is too cold (≤15°C)**(incorrect measurement!).**

Curve C:
The sample being used is too warm. (≥ 21°C). The drop in pressure results from the overlapping of "BOD" and volume contraction **(incorrect measurement!)**

The initial phase of the measurement must take no longer than 3% of the actual measurement duration. For this reason, the OxiTop® system starts the BOD_5 determination after three hours at the latest. Therefore, the bottled measurement sample must not be less than 15°C as, otherwise, there is insufficient time for a temperature adaptation.

Special tip and to recapitulate:

The OxiTop® system can differentiate between a negative pressure from the BOD measurement and an overpressure resulting from the increase in temperature. However, it cannot differentiate between the negative pressure resulting from the BOD process and the negative pressure caused by the decrease in temperature! Therefore, the sample should not be warmer than 21 °C. One degree Celsius temperature adaptation can be assimilated during the adaption phase without any problem.
If the measurement sample is more than 21°C, this results in a BOD value that is too high!
If the sample is less than 15°C, the maximum allowable initial phase time is no longer sufficient. The measurement must be started although the BOD value will be marred by a low result.

Evaluation of the measurement

OxiTop® system

(Note: The OxiTop® Control system is covered following)

Automatic storing of measured values

Beginning with the start time of the measurement, the OxiTop® automatically stores one value every 24 hours. The individual measured values can be called up by actuating the push button, "S". In this case, the number of the measured value that corresponds to the day (1-5) appears first and is followed by the stored value. This is possible both during the measurement as well as after it.
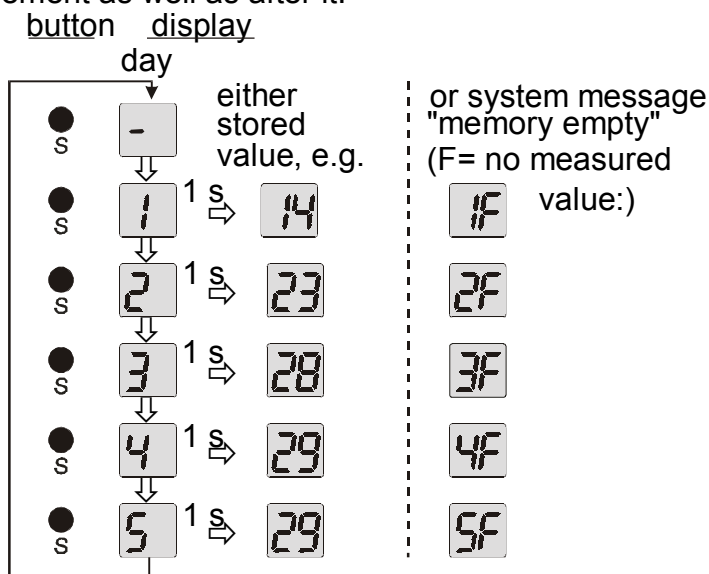
The values are stored until the measuring head is restarted. As a result, the daily recording of values, particularly at the weekend, is not necesarry.

BSB curve from individual measurements
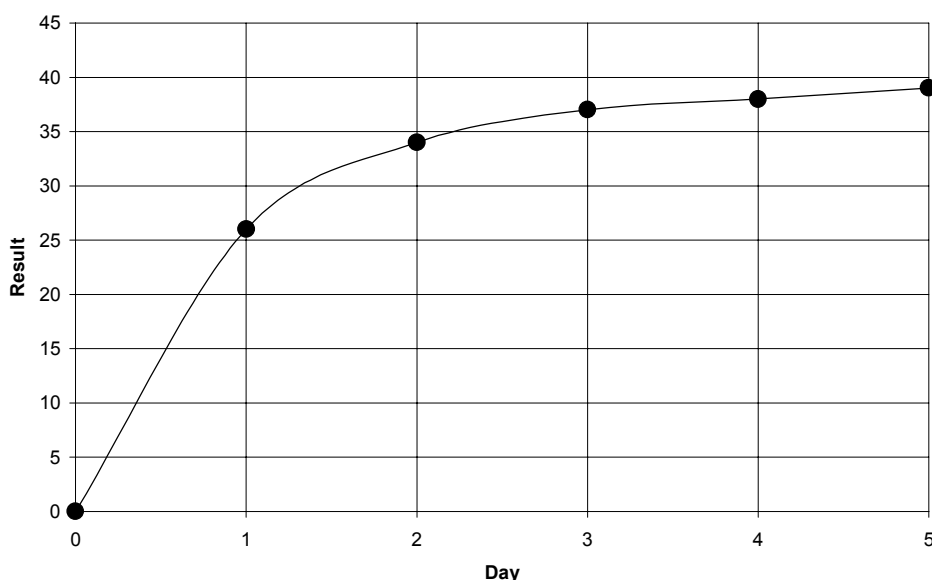
Measurement results

A value is stored every 24 hours for the BOD_5 determination using the OxiTop$^{®}$ measuring system. The display is in digits. The conversion into actual BOD values is performed by multiplying with factors and, as a result, subject to the sample volume. After 5 days, 5 values have been stored. Entered on an evaluation sheet or on graph paper, they reproduce the oxygen degradation curve of the sample. The fifth value is the required BOD_5 value.

Graphical evaluation has the advantage that the type of depletion can be recognized more easily. It is often readily apparent from the five daily values whether, for example, an inhibition or nitrification was present in the measurement of the sample.

The necessary factor is calculated according to the BOD equation:

$$BOD = \frac{M(O_2)}{R \cdot T_m} \cdot \left(\frac{V_{tot} - V_I}{V_I} + \alpha \frac{T_m}{T_0} \right) \cdot \Delta p(O_2)$$

$M(O_2)$	Molecular weight of oxygen (32000mg/mol)
R	Gas constant (83.144 L·mbar/mol·K)
T_0	Reference temperature (273.15 K)
T_m	Measuring temperature (293.15 K)
V_{tot}	Bottle volume (theoretical volume) [mL]
V_I	Volume of sample [mL]
α	Bunsen absorption coefficient (0.03103)
$\Delta p(O_2)$	Difference of the partial oxygen pressure [hPa]

In order to be able to make a calculation with even-numbered multiplication factors, the starting volumes are adapted to the equation mentioned above. This is the sole reason for the certainly rather unusual volumes of 432 mL, 365 mL, etc.

At this point, the measuring principle should be mentioned again. The OxiTop$^{®}$ measures the difference in pressure and calculates the BOD according to the equation mentioned above.

As a result, the experimental conditions must fulfill the prerequisites of the equation! That is to say, the bottle volume must be 510 mL, an allowable filling volume (432 mL, 365 mL, 250 mL...) must be used and the measuring temperature must be 20°C.

If this is not the case, an incorrect BOD value will inevitably result.

For applications that go beyond routine determinations, there is the "BOD Special" mode in the OC 110 Controller. These parameters are employed as variables in this mode and different values can be entered explicitly, e.g. a measurement at 27°C in a 1000 mL bottle with a filling volume of 277 mL. The controller then performs the calculation with precisely these values.

BOD Primer

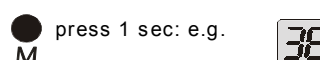## Calling up manually measured values

In order to recognize the method of depletion and possible inhibitions or nitrification better and also more rapidly, it is recommended to determine frequently measured individual values directly at the beginning of a measurement. At any point of time during the measurement, the current measured value can be called up by actuating the push button, "M".

start measurement: **S** and **M** press

`--` 2 s ⇨ `00` stored values are deleted

momentary value: **M** press 1 sec: e.g. `38`

stored values: **S** press 1 s see "measurement":
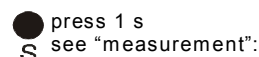
This value provides information on the current status of the measurement. The display switches itself off again afterwards.

A further measured value can be recorded at any time, e.g. 7 days later or 28 days later. The only limit is governed by the oxygen content within the measuring system. If the oxygen is depleted, the maximum negative pressure is reached. If this occurs during a measurement, the range of measurement is wrongly selected and the measurement must be repeated.

`--` The OxiTop® measuring head shows "overflow".

BOD curve of individual measurements

😊 Manually measured individual values

● Automatically stored values
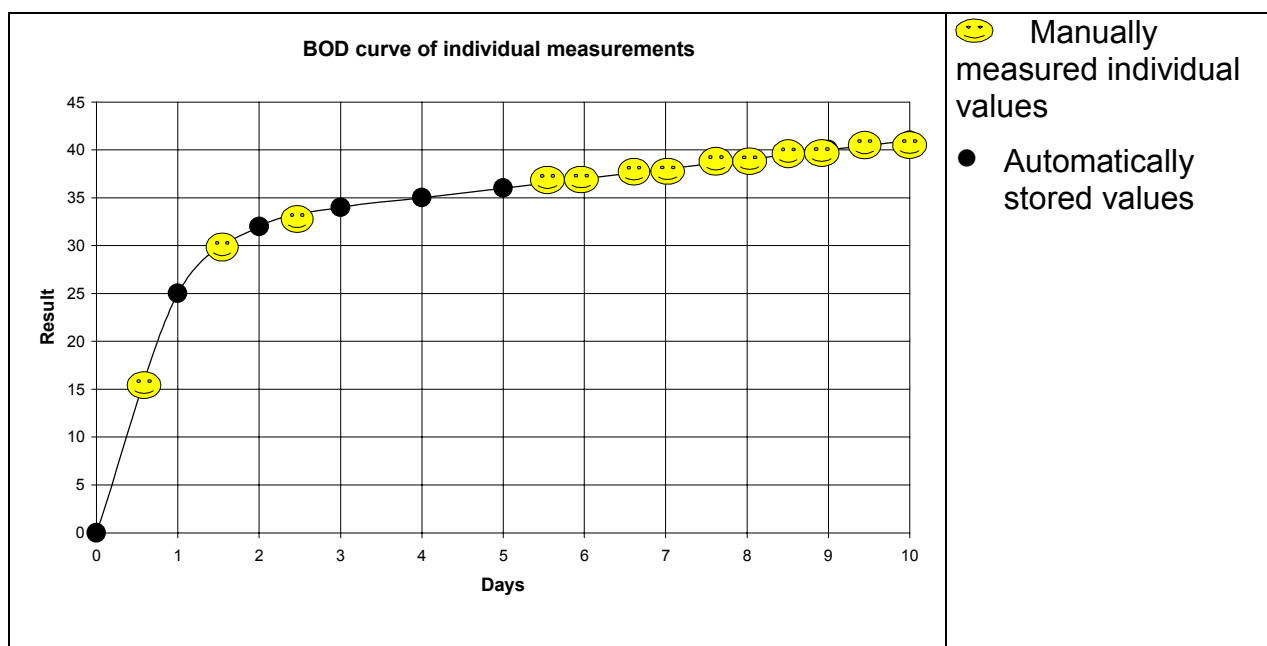
The OxiTop® measuring head continues to measure until the measurement is restarted.

OxiTop® Control system

The OxiTop® Control system records 180 to 360 measured values depending on the total measuring duration (0.5 hours to 99 days). For example, there are 360 individual measured values in the BOD_5 measurement. This enables a detailed examination of the measured curve. This measuring system also enables average value determinations of parallel samples with statistical evaluation. As a result of its various measuring and setting options, the OxiTop® Control system is also suitable for research tasks and degradation tests to name but two examples, as well as for classical BOD_5 measurement.

The OxiTop® controller communicates with the OxiTop® Control measuring heads via an infrared interface. The controller performs all of the sample management including data storage and graphical evaluation. The RS 232 interface is used to conveniently transmit the stored data to a PC using the "Achat OC" program.
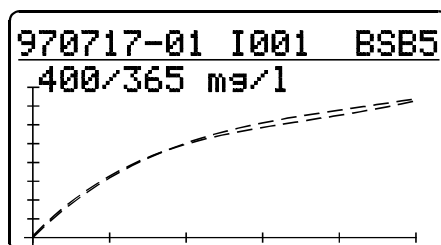
Graphical representation of a parallel determination of the BOD_5 on the controller.

The prevailing pressure inside the bottle (quantity to be measured!) is affected by the temperature fluctuations of the incubator. Each time the thermostat door is opened, a fluctuation in temperature occurs. In order to avoid this, thermostatic cabinets with glass doors are available. Because the data transmission between measuring head and controller is performed using infrared beams, this is also possible when the glass doors are closed. This reduces external influences to a minimum.

Examples of BOD graphs

The following graph shows some examples of characteristic BOD curves.
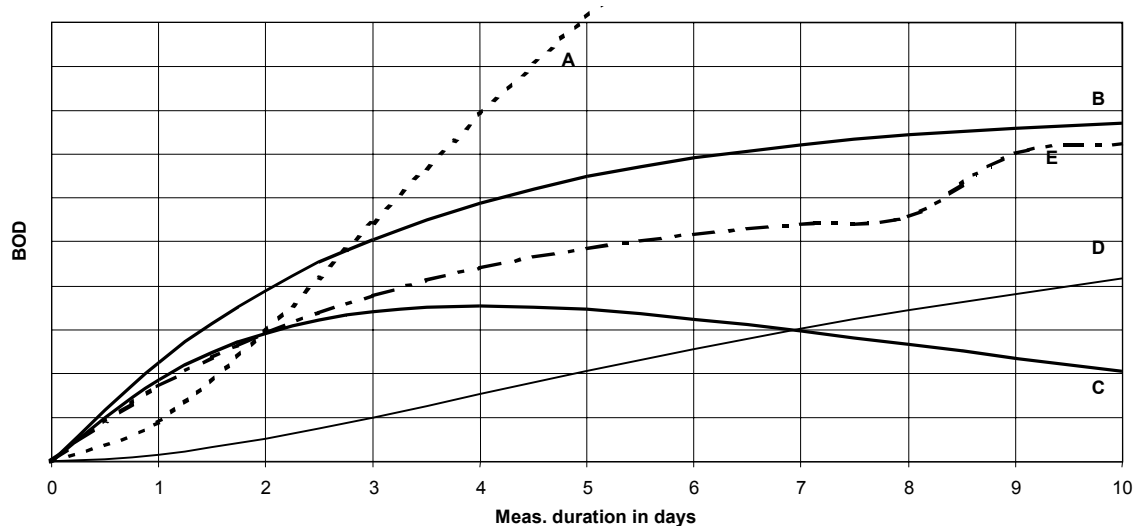
Curve A   The BOD_n value is too high, the oxygen content in the bottle was not sufficient. The sample must be diluted or another measurement range selected.

Curve B   Normal course of a BOD_n graph.

Curve C   The system did not provide any correct results for the BOD_n calculation. Possible causes: inadequate seeding with microorganisms, leaks, no dosage or too low dosage of NaOH pellets, etc.

Curve D   The bacteria could not (or could only badly) adapt themselves to the specified environmental conditions, or the seed, i.e. the addition of microorganisms, was insufficient.

Curve E   An unwanted process occurred, e.g. unwanted nitrification.

Control measurements

Control measurements are used to check the measurement or the equipment employed. The OxiTop® measuring system provides three possible types of check for the respirometric BOD measurement.

Standard solution according to *Standard Methods 5210 D*

The measurement can be checked using a glucose/glutamic acid solution (Appendix R10). The theoretical BOD_5 value of this solution lies at 307 mg/L. Between 75% and 94% of the theoretical BOD value is biologically degraded. The glucose/glutamic acid solution really must be seeded as, otherwise, no microorganisms will be present in the solution. That is the reason why this type of check is the most time consuming. The standard solution must be prepared with seeded dilution water and the BOD value of the seeded dilution water separately determined in order to compensate for its contribution. A brief overview of the measurement follows:

The required measuring volume for the standard measurement is 164 mL. The seed, all the minerals, nutrients and trace elements are added in sufficient quantity with the dilution water.

In order to take account of the oxygen consumption of the dilution water, 432 mL of the dilution water must be measured in parallel as a control measurement. The BOD_5 value of the dilution water must not exceed 2 mg/L.

The result is then calculated according to the following equation

$$BOD_n = \left[A - B \cdot \frac{V_t - V_e}{V_t} \right] \cdot \frac{V_t}{V_e}$$

where:
A   Measured value of the diluted standard solution after n days [mg/L]
B   Measured value of the seeded dilution water after n days [mg/L]
V_e Volume of sample [mL] that was used for the production of the respective analysis solution
V_t Total volume [mL] of this analysis solution

Note: This calculation formula differs somewhat from the equation specified in the *Standard Methods* as the OxiTop® system already provides a concentration result [mg/L]. However, the original formula is calculated with the oxygen absorption in mass units [mg].

The result of the measurement of the standard solution with glucose/glutamic acid standard should be 260 ± 30 mg/L.

BOD5 curves for the glucose/glutamic acid standard solution
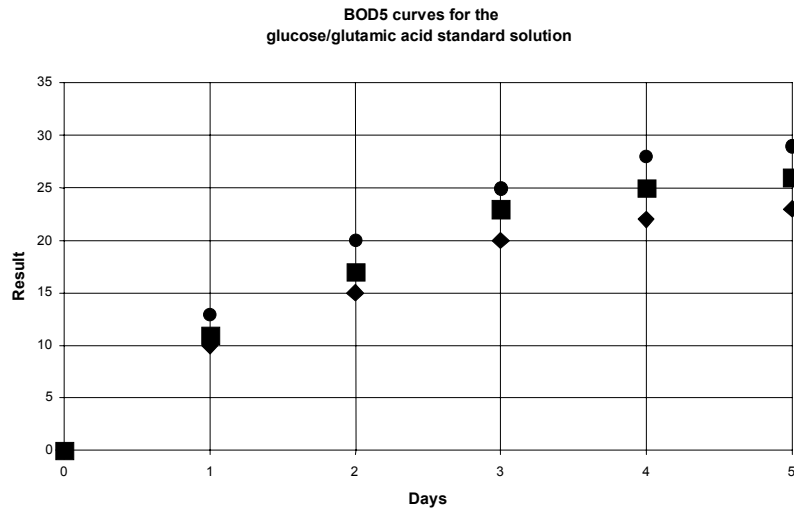
The advantage of this check lies in the fact that, in this case, not only the measuring equipment is checked, but also the efficiency of the biology employed.

Calibration tablet OxiTop® PM

The OxiTop® PM calibration tablet is made up of a precisely defined quantity of chemicals which react with dissolved oxygen and, as a result, abstract oxygen from both the gas phase and the liquid phase. The result is a negative pressure that corresponds to a BOD and can be used for checking the OxiTop® system. The specified theoretical value must be achieved and then held for five days.
This way it is possible to check the operation and sealing of the entire OxiTop® system.
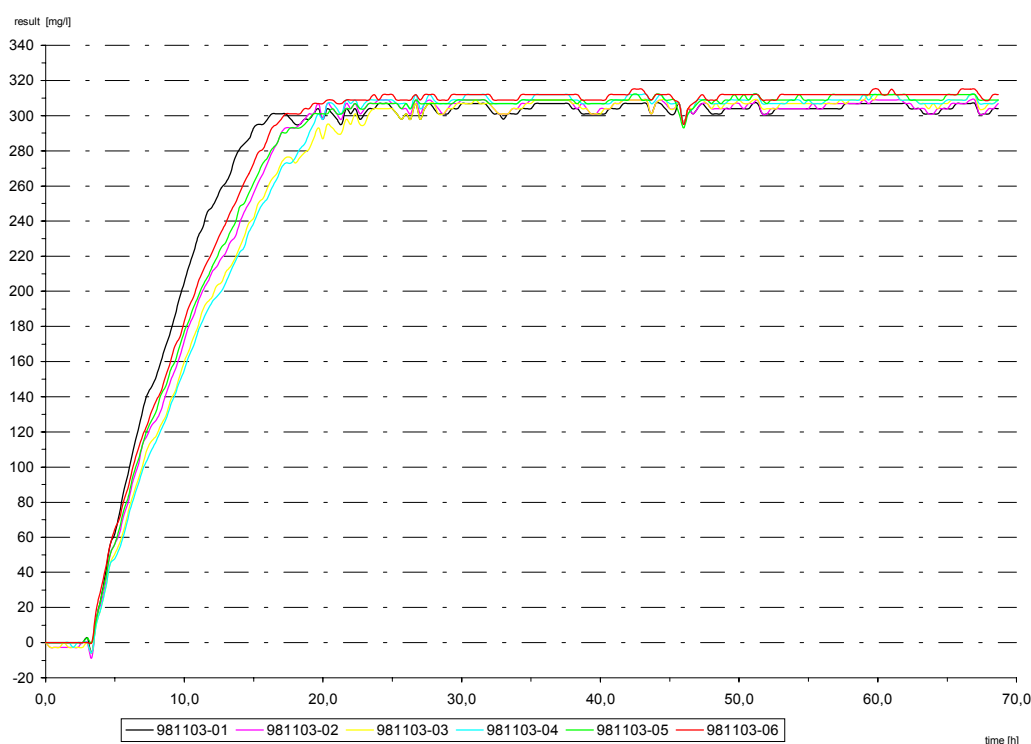
The calibration tablet cannot be used to check the efficiency of the biology, but can be used to check the operation of the entire measuring apparatus. Moreover, the experimental requirements in comparison to the check using the glucose/glutamic acid standard solution are extremely low as the following instructions clearly illustrate.

Instructions for checking with the OxiTop® PM (calibration tablet)

1. Set the thermostat cabinet to $(20 \pm 0.5)°C$ and connect the stirring platform.
2. With the aid of an overflow measuring flask, pour 164 mL distilled water into a BOD bottle and insert a magnetic stirrer bar.
3. Place the bottle on the stirring platform in the thermostatic cabinet and switch on the stirrer.
4. Start the measuring heads to be checked and place them individually in the thermostatic cabinet.
   The oxygen consumption of the calibration tablet proceeds unusually rapidly. For this reason, the AutoTemp function must be avoided.
5. Thermostat the measuring heads and bottles for 4 - 4.5 hours in the thermostatic cabinet.
   During this thermostatting time, the AutoTemp time interval expires and no longer affects any subsequent measurement!
6. After the thermostatting time, add a calibration tablet to each bottle.
7. Insert the rubber sleeve without any absorber (e.g. NaOH) as a sealing ring.
   This involves a chemical reaction in which no carbon dioxide is set free which is also why no NaOH is required!
8. Immediately screw on the OxiTop® measuring head and close it tightly. Under no circumstances should you restart the measurement as, otherwise, the AutoTemp interval would begin again!
9. The measurement runs and is stirred for the next five days in the thermostatic cabinet.
10. Compare the measured value with the expected theoretical value (specified on the package) and enter it on the log sheet provided.

If the measured value agrees with the theoretical value that is specified on the package, this confirms that the total OxiTop® system is operating correctly and, as a result, is ready for use.

Wertetabelle / Table of values

Meß-system Measuring system	Proben-Nummer Sample number	Produktbezeich-nung und Charge Product designation and lot	Start-datum Date of start	1. Tag 1st day	2. Tag 2nd day	3. Tag 3rd day	4. Tag 4th day	5. Tag 5th day	Bemer-kung Remark	Chargen prüfwert Lot test value

If all the test values are entered in this table, a measuring system can be monitored over a long period of time. Only in this way can long-term changes actually be detected.

OxiTop® PT testing agent (to check the measuring head)

The OxiTop® PT testing agent consists of an appliance which can be used to produce a defined negative pressure. This negative pressure is dependent on the altitude above sea level (atmospheric air pressure) at which the check is performed since the air pressure at the Dead Sea is higher than on Mount Everest.

The application is the rapid checking of the OxiTop® and OxiTop® Control system for correctness of the pressure measurement.
A long-term check of the tightness of the OxiTop® or OxiTop® C system bottle is not possible with this.

Checking the OxiTop® system

Only use the original rubber sleeves of the OxiTop® PT! Otherwise, measurement errors are possible.

Insert a rubber sleeve in the OxiTop® PT test device.
Position the plunger of the syringe at the 5th scale mark on the scale (0.5mL).

While doing so, the OxiTop® must not yet be screwed onto the testing device.

Tightly screw the OxiTop® onto the testing agent.
Press the "S" and "M" keys at the same time for 2 seconds.

OxiTop® must display $\boxed{00}$.
Pull out the plunger to the 20th mark on the scale (2mL).
Press the "M" key and read the measured value.
Find out the height above sea level and use the relevant control value from the table.

Reading example:
Location: WTW Weilheim / height above sea level:
565 meters / nearest altitude value in the table: 600 meters
Control value: 36 digits
Determine the deviation   (measured value – control value).
The deviation must not be more than +/-3 digits.
The normal fluctuations of air pressure are taken into account.
Checking the OxiTop® Control system:
See Operating Manual for the OxiTop® Control System, Chapter GLP/TOOLS – Check – Pneum. Test.

Höhe über Meeres spiegel (NN) [m]	Mittlerer Luftdruck [hPa / mbar]	Prüfwert [Digit]
Altitude above sea level [m]	*Average air pressure [hPa / mbar]*	*Control value [Digit]*
-300	1 050	41
-200	1 037	40
-100	1 025	40
0	1 013	39
100	1 001	39
200	989	38
300	977	38
400	966	37
500	954	37
600	943	36
700	932	36
800	921	36
900	909	35
1000	898	35
1100	888	34
1200	877	34
1300	866	33
1400	856	33
1500	845	33
1600	835	32
1700	825	32
1800	815	31
1900	805	31
2000	795	31
2100	785	30
2200	775	30
2300	766	30
2400	756	29
2500	747	29

Cleaning and Maintenance

Cleaning

The graduated measuring flasks, magnetic stirrer bars and rubber sleeves must be cleaned each time they are used. However, do not use detergent. Traces of detergent in the bottle or on the magnetic stirrer bars can lead to incorrect measurements as they can affect the biology. In any case, traces of detergent in the bottle would contribute to the BOD value!

! Mechanical cleaning with a brush and rinsing with dilute hydrochloric acid have proved to be suitable. (Follow the safety instructions.) Afterwards, ensure that any remaining acid is completely removed (e.g. by measuring the pH value).

The OxiTop® measuring head does not come into contact with the measurement solution during the measurement and, thus, does not require regular cleaning. Any splashes on the case can be removed with a cloth.

Maintenance

The OxiTop® system is battery operated. If "LO" appears on the display, replace the Lithium batteries. The more seldom data are called up, the lower the energy consumption of the battery and the longer the battery lasts!

IF	Memory empty (IF=measured value of day 1 is missing)	--	Value remains below measuring range
LO	Change batteries (approx. every 3 years)	-_	Value exceeds measuring range > 50 digit

BOD_n determination according to DIN EN 1899-1 (ISO 5815), DIN EN 1899-2 and Standard Methods 5210 B

Water samples from 3 mg/L to 6000 mg/L BOD can be analyzed using the BOD determination according to the Euronorm DIN EN 1899-1 (identical with ISO 5815). At the same time, this is also the reason why samples must be diluted at all. Oxygen saturated water has an oxygen concentration of approx. 9 mg/L. If the sample now has a BOD_n of 5000 mg/L, it is easy to see that the oxygen content in the graduated measuring flask is not sufficient.

As a result, a prerequisite on the dilution water to be used becomes immediately apparent, i.e. it must be oxygen saturated. With dilution, the concentration of oxygen consumption is lowered so far that a measurement is possible and, also, lowered so far that 9 mg/L dissolved oxygen in the bottle is sufficient. As a result, is also clear why the dilution depends on the expected BOD. The Standard Methods 5210 B (5-day BOD test) describes the so-called dilution BOD, too. The differences to the Euronorm are insignificant.

A BOD_n method that manages without dilution and in which the oxygen content is also determined by means of amperometric sensor or iodometric titration is described by the DIN EN 1899-2. In this case, however, the allowed BOD lies between 0.5 and 6 mg/L. With that, the oxygen concentration in the bottle is sufficient if the sample is saturated and no dilution is required.
The most important aspects of the determination of the BOD_n are now addressed in the following section.

Dilution method (DIN EN 1899-1, ISO 5815, Standard Methods 5210 B)

Analogous to the respirometric determination of the biochemical oxygen demand, the dilution method measures the consumption of oxygen related to the biological activity. The BOD_n is represented as the difference of the oxygen concentration at the beginning of the incubation and after the n-day incubation under consideration of the respective dilution ratios as well as of the blank value of the dilution water.

Basics

In this case, the measurement operates not via the pressure but directly via the determination of the dissolved oxygen that is determined using an amperometric sensor or iodometric titration complying with standards according to DIN EN 1899-1. In respirometric BOD, the oxygen is also drawn from the headspace above the sample. For the dilution method, the effect of a gaseous phase is precluded because in the same way as in the equation cited for respirometric BOD, $p(O_2)_{fl} = p(O_2)_g$, the

concentration of the dissolved oxygen would be changed. The consequence of this is very simple. Karlsruher or Winkler bottles (or Wheaton bottles) must always be completely full! No gas bubbles must be present in the bottle!
The respiration process naturally remains unaffected by this. Oxygen is inhaled and carbon dioxide exhaled. However, both gases remain dissolved.

Brief instructions on how to perform a measurement

1. Estimate the range of measurement of the sample to be analyzed

2. Produce the dilution water with the required electrolyte additives

3. Aerate the dilution water

4. Add the seed (ideally with adapted biology)

5. Produce the analysis solutions according to the expected BOD_n with the addition of nitrification inhibitor

6. As recommended, prepare several dilutions in a geometrical progression that encloses the dilution with the expected BOD

7. Prepare a blank test determination of the seeded dilution water to which nitrification inhibitor is also added

8. Pour the analysis solutions and blank test solutions into Karlsruher or Winkler bottles (alternatively, Wheaton bottles) as repeat determinations (two analog series)

9. Determine the oxygen concentration in one of the series

10. Close the bottles with stoppers so that no air bubbles are included

11. Incubate the samples for n days at 20°C

12. After incubation, determine the final oxygen concentration in all the analysis solutions and in the blank test solutions

13. Calculate the BOD_n values and then the average value for the sample

Components of the measuring system for determining the dilution BOD

Karlsruher or Winkler bottles (or, alternatively, Wheaton bottles)
The funnel-shaped bulge in the neck of the bottle enables the oxygen sensor to be submerged without causing the measuring sample to overflow. The sample displaced by the sensor collects in the neck of the bottle and runs back into the bottle when the sensor is removed.

Oxygen sensors
Amperometric sensors are used for measuring the dissolved oxygen (see Oxygen Primer). During this process, it is important that sample is constantly fed to the membrane. The StirrOx® G sensors specially developed for BOD measurement are equipped with a type of propeller on the shaft that fulfills this task. If sensors such as the CellOx® 325 or TriOxmatic® 300 are used, we recommend using the RZ 300 attachment stirrer. This is plugged directly onto the probe, has the same diameter and, therefore, fits exactly into the neck of the bottle. In this case, it is important to note that an Oxi-Stirrer 300 should be used instead of a magnetic stirrer because the RZ 300 is moved by an alternating electromagnetic field. Normal magnetic stirrers, on the other hand, have a rotating counter magnet.

Nitrification inhibitor
In the same way as the respirometric BOD determination, the processes of the nitrification bacteria must also be suppressed here. The measurement solution has nitrification inhibitor added to it to suppress the conversion of ammonium to nitrite and then to nitrate.

Incubator

In the same way as the respirometric measurement, the samples must be stored at 20 ± 1 °C for the n days of the BOD determination. In this case, it is important to note that the incubator cabinet does not have glass doors as the sample bottles are made of white glass and the light could cause changes to the sample. This would result in the growth of algae. Otherwise, however, there is no difference in the storage of the sample bottles.
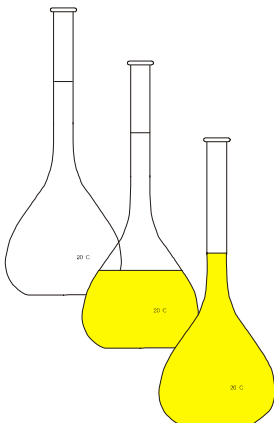
Measuring flasks and pipettes

Calibrated measuring flasks and pipettes are used to produce the necessary dilution solutions. However, when pipettes are being used, it is important to watch out that no flakes or larger particles block the tip of the pipette during suction. This effect would then lead to a type of microfiltration and the measurement sample would produce a result that is too low. This danger is greater if the pipetted volumes are very small. In order to avoid this effect in respirometric analysis, overflow measuring flasks are used.
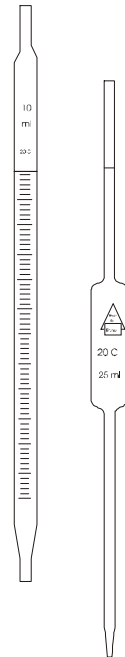
Measurement

Dilution water

In the production of the dilution water required for the measurement, a differentiation must first be made between the dilution water and the seeded dilution water.
The basis is the use of distilled water (Appendix A). That is surprising at first glance because, as already mentioned several times, distilled water damages bacteria as a result of osmotic processes. In addition, DIN 38409 part 51 (that is no longer valid) stipulated drinking water and distilled water was expressly forbidden. The background to this is as follows. In the old norm, only a few additional chemicals (merely urea and pentasodium triphosphate) are added. In the current standard method, however, a multitude of chemicals is added so that a type of "standardized drinking water" is created from the distilled water. As a result of the addition of electrolyte, the damaging osmosis no longer takes place.
Now, 1 mL each of phosphate buffer solution (Appendix V1), magnesium sulfate heptahydrate solution (Appendix V2), calcium chloride solution (Appendix V3) and ferric (III) chloride hexahydrate solution (Appendix V4) are added to 500 mL distilled water then diluted to 1000 mL and aerated for at least one hour with suitable equipment. In order to avoid oxygen supersaturation, leave the aerated dilution water to stand open for one hour and use it within 24 h.

The Euronorm DIN EN 1899-1 now allows the following materials to be used as seed sources:
- Municipal wastewater
- Surface water
- Settled effluent from a wastewater treatment plant
- River water that was taken in the main channel downstream
- Commercially available seed preparation

The user must realize that, according to the seed used, more or less bacteria are present that are also adapted to a greater or lesser degree. As a result, the BOD value is also dependent on the seed material. Therefore, the *Standard Methods 5210 B* recommends the wastewater of the plant that also cleans the wastewater due to the adapted biology. The best results are usually obtained if water from the effluent of the preclarification is used after it has settled out.

To recapitulate:

Different seed sources can lead to different BOD results. The numerically largest BOD values are usually reached with seed from the influent to the biological purification stage of the plant that purifies the wastewater of the sample. If other seeds are used, the values are usually lower due to the less adapted biology as the degradation performance is lower.

According to its origin, 5 to 20 mL seeding water is now added to each liter of the dilution water. The seeded dilution water should only ever be prepared immediately before the measurement (and used only for this laboratory day).

Ensure that the measurement sample to be analyzed has a pH value between 6 and 8 (can be adjusted with diluted hydrochloric acid or sodium hydroxide solution, if necessary) and that no free or bound chlorine (can be removed with sodium sulfite solution) is present.
With regard to sampling and homogenization, refer to the section on respirometric measurement. The points cited there also apply here.

The typical dilutions that are prepared depend entirely on the expected BOD value. Samples with a high BOD must be heavily diluted so that the oxygen in the Karlsruher or Winkler bottles is sufficient. Samples with a low BOD must be less heavily diluted. The DIN EN 1899-1 recommends the following dilution ratios where the dilution factor describes the quotient obtained from the volume of diluted sample divided by the volume of the analysis sample:

| Expected BOD [mg/L]	Dilution factor	Examples of water
3 – 6	1.1 – 2	River water
4 – 12	2	River water, biologically purified municipal wastewater
10 – 30	5	River water, biologically purified municipal wastewater
20 – 60	10	Biologically purified municipal wastewater
40 – 120	20	Purified municipal wastewater or slightly polluted industrial wastewater
100 – 300	50	Purified municipal wastewater or slightly polluted industrial wastewater, municipal raw wastewater
200 – 600	100	Purified municipal wastewater or slightly polluted industrial wastewater, municipal raw wastewater
400 – 1200	200	Heavily polluted industrial wastewater, municipal raw wastewater
1000 – 3000	500	Heavily polluted industrial wastewater
2000 – 6000	1000	Heavily polluted industrial wastewater

The following points must now be noted in the production of the analysis solution:
The sample should have a temperature of $20 \pm 2°C$ and is already in the dilution vessel. Afterwards, the nitrification inhibitor solution is added and it is filled up to the calibration mark with the seeded dilution water according to table mentioned above. Carefully mix the solution to avoid the inclusion of air bubbles and then pour it into the Karlsruher or Winkler bottles.

Since the precisely correct degree of dilution is difficult to achieve, it is better to prepare a series of dilutions that has the expected value in the middle. As a rule of thumb for this purpose, the following guideline applies: prepare five different dilutions for unknown samples and three different dilutions for known samples in order to perform repeat determinations.
Moreover, do not forget the blank test determination of the seeded dilution water under any circumstances. Only ATU is then added to the dilution water in order to suppress nitrification.

Note: DIN EN 1899-1 only requires a repeat determination in the oxygen determination using iodometric titration because the first series after the initial concentration determination is discarded and only the second series is incubated.

Oxygen concentration determination
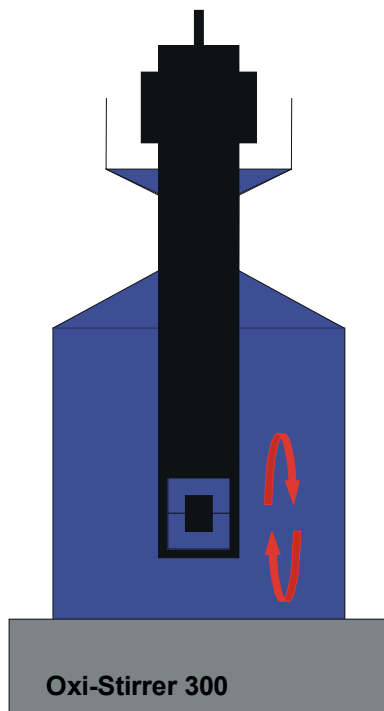
The various sample solutions are subsequently transferred into Karlsruher or Winkler bottles. This must be done carefully in order to avoid the inclusion of air bubbles. It is best to let the solution run down the bottle walls similar to the way in which a beer is poured. The solution should then reach the lower edge of the funnel. Now, the sensor is inserted into the bottle. The displaced sample collects in the funnel of the bottle. After the measured value has been recorded (do not forget the approach flow of the sensor during the measurement!), the sensor is pulled out, the sample runs back and the stopper is inserted and, namely, in such a way that no more air is found in the sample bottle!

Oxi-Stirrer 300

All the samples must be free of air bubbles when they are poured into the bottles!
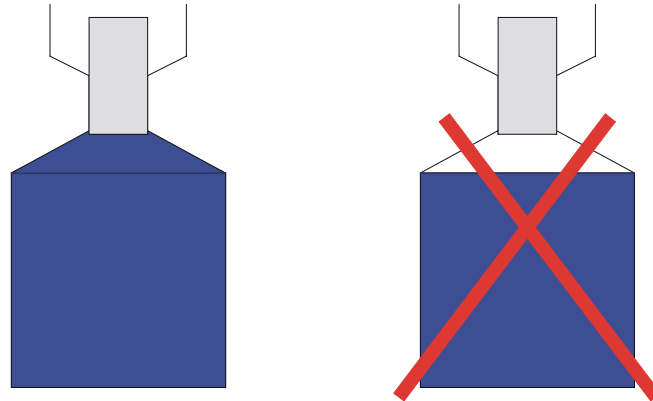
The sample bottles are subsequently incubated for n days ± 4h at 20°C in the dark. The darkness is particularly important in this case as the Karlsruhe or Winkler bottles (or Wheaton bottles) are mostly made of white glass. A growth of algae could occur in the bottles in an incubator with glass doors.

After incubation, the oxygen concentration in the bottles is determined again. To do this, the measurement must be started immediately the stopper is pulled out. If the bottles are open, oxygen from the atmosphere can diffuse in and falsify the result. The submersed sensor acts as a stopper during the measurement which causes this effect to be suppressed during measurement.

Evaluation of the measurement

While, in DIN 38409 part 51, the value of the BOD was determined by plotting the oxygen values against the dilutions in a graph or from the corresponding linear regression, the BOD for each sample bottle is determined according to DIN EN 1899-1 and, afterwards, an average value formation is performed. The BOD for each sample is calculated according to following equation (the calculation according to Standard Methods 5210 B is performed in the same way):

$$BOD_n = \left[(c_1 - c_2) - \frac{V_t - V_e}{V_t} \cdot (c_3 - c_4) \right] \cdot \frac{V_t}{V_e}$$

where:

c_1 Concentration of dissolved oxygen [mg/L] in an analysis solution at zero time

c_2 Concentration of dissolved oxygen [mg/L] in the same analysis solution after n days

c_3 Concentration of dissolved oxygen [mg/L] in the blank test solution at zero time

c_4 Concentration of dissolved oxygen [mg/L] in the blank test solution after n days

V_e Volume of sample [mL] that was used for the production of the analysis solution in question

V_t Total volume [mL] of this analysis solution

Control measurements

The Euronorm DIN EN 1899-1 and the norm of the Standard Methods 5210 B recommend a control analysis in each sample series to check the seeded dilution water, the seeding water and the technique of the analyst. This check is performed again with glucose-glutamic acid standard solution.

20 mL of the glucose-glutamic acid standard solution (Appendix V5) are added with ATU solution and diluted to 1liter with seeded dilution water. The further procedure corresponds to the standard measurement. The BOD values obtained in this way should result in 210 ± 40 mg/L for the BOD_5 measurement and 225 ± 40 mg/L for the BOD_7 measurement.

Procedure for undiluted samples (DIN EN 1899-2)

Basics

The Euronorm DIN EN 1899-2 is suitable for samples with a BOD that lies in the range of between 0.5 and 6 mg/L. The concentration of oxygen-saturated water is approx. 9 mg/L. As a result, the oxygen present in the sample is sufficient for such low BOD values. In this case, the principle is extremely simple. The original sample is poured into a Karlsruher or Winkler bottle, the oxygen concentration is measured, incubated for n-days at 20°C, and the oxygen concentration is measured again. The difference then corresponds to the BOD_n.
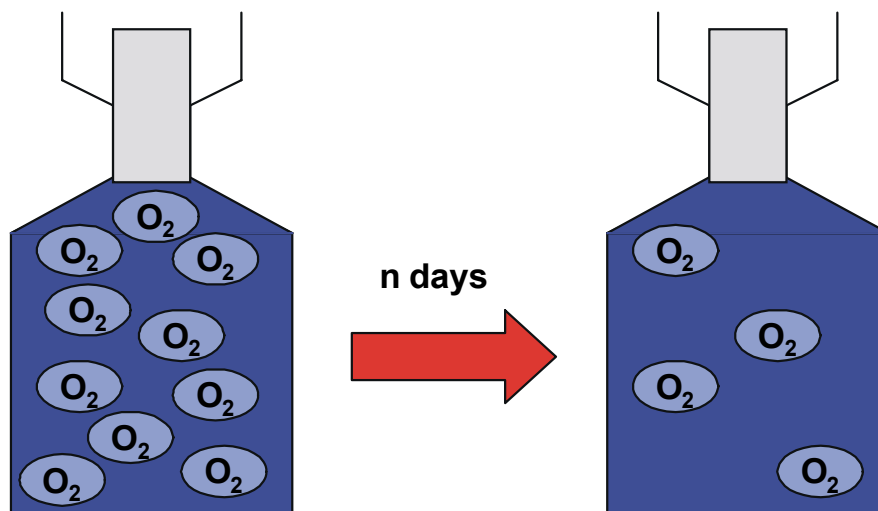
A special explanation of the brief instructions and the equipment employed is not required at this point, as this is analogous to the dilution method already described. The DIN EN 1899-2 norm deals more or less with a determination according to the dilution principle, just without the dilution. The notes on sampling and homogenization should also be followed.

Measurement

The water sample to be analyzed must be thermostatted to 20°C and be saturated with oxygen. DIN EN 1899-2 recommends aeration and then leaving the open sample to stand for 15 min to avoid oversaturation. The sample is subsequently poured into Winkler or Karlsruher bottles whereby no air bubbles may be included. In order to suppress the nitrification, the addition of ATU is allowed according to DIN EN 1899-2. After the oxygen concentration determination, the analysis samples are incubated at 20°C for five or seven days. The incubation must be made with light excluded in order to avoid effects that result from the growth of algae. After the incubation period, an oxygen concentration determination is performed again and delivers the required final value. The oxygen determination is performed analogous to DIN EN 1899-1 with the oxygen sensor or iodometric titration. At the same time, the notes provided in the previous section should be followed.

Evaluation of the measurement

The calculation of the values sought for biochemical oxygen demand turns out to be very simple because it represents the difference between the initial and final determination of the oxygen concentration.

$$BOD_n = (c_1 - c_2)$$

where:
c_1 Concentration of dissolved oxygen [mg/L] in an analysis solution at zero time
c_2 Concentration of dissolved oxygen [mg/L] in the same analysis solution after n days

Note: If, in the equation for calculating the dilution BOD, the volume of sample V_e is set to the same value as the total volume V_t, the equation shown above is achieved. This is logical, as no dilution has taken place.

One point must be added. The sample must contain sufficient bacteria for this determination. Obviously, toxic industrial wastewater cannot be analyzed using this method.

BOD$_n$ cuvette test

The determination of the biochemical oxygen demand using cuvette test sets depends heavily on the dilution BOD according to DIN EN 1899-1. The dilution water employed corresponds practically to the demands of the standard method, but not to the method of the determination of the oxygen concentration. The dilution BOD according to DIN EN 1899-1 calls explicitly for the use of either the iodometric titration or the amperometric oxygen sensor.
In the BOD cuvette test, however, the concentration determination is performed photometrically. As a result, the cuvette test is a self-control measurement not a method that complies with standard methods, even if this is sometimes so presented by diverse suppliers.

The determination with the cuvette test set 00687 is performed as follows. The diluted sample and the dilution water must be analyzed for their oxygen content before and after the incubation. One part is used for the initial determination and is then discarded, the remainder is incubated in the appropriate vessels at 20°C for 5 (or n) days and then used for the final concentration determination.
As a result, a total of four photometric determinations are required for the BOD measurement result.

Basics

The background of photometry is a chemical reaction that transforms the substance to be determined into a colored compound whose intensity is then determined by a photometer. The color intensity corresponds to the wanted concentration.
All the required reagents are included in the test set.

For the oxygen determination, the following chemical reaction starts:

The oxygen that is present in the sample oxidizes the manganese

$$4\ Mn^{2+} + O_2 + 4\ H^+ \rightarrow 4\ Mn^{3+} + 2\ H_2O$$
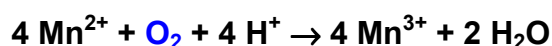
The resulting manganese (III) forms a red colored complex with the titriplex II

$$Mn^{3+} + Titriplex\ II \rightarrow \textbf{red dye}$$

A red colored dye mainly absorbs light in a wavelength range of 500 nm. For this reason, light with a wavelength of 500 nm or 525 nm is used for the photometric measurement according to the type of photometer.
If there is now a lot of oxygen in the sample, a correspondingly large amount of the red complex compound develops and the coloration is very intense. If there is little oxygen in the sample, the color development that appears is very pale or colorless. In the spectrum shown below, the first case is represented by the dark red curve and the second case represented by the light red curve. In the range of 500 nm, in the one
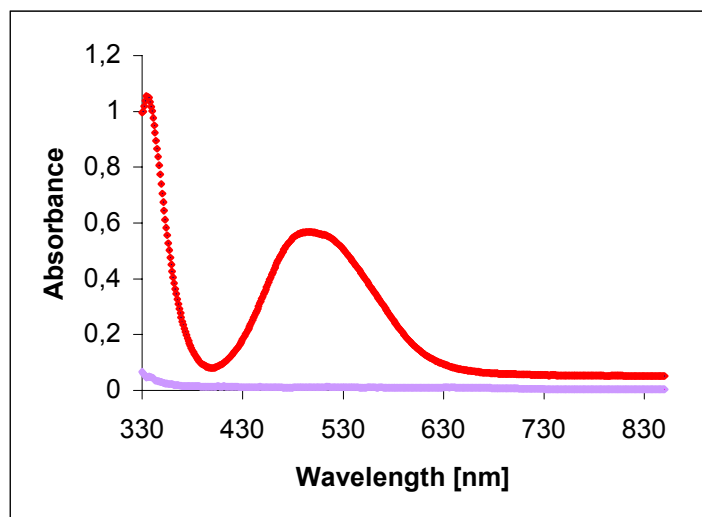
case, a great deal of light is absorbed (higher absorbance value) and, in the second case, very little is absorbed (very low absorbance value). The absorbance value is the quantity of light absorption to be measured.

From these measured absorbance values, the photometer can calculate the oxygen concentrations by means of stored calibration curves and these can then be used to calculate the BOD value.

Brief instructions on how to perform a measurement

1. Estimate the measuring range of the samples to be analyzed

2. Produce the seeded nutrient solution with settled wastewater, ideally from the effluent preclarification, the contents of the small bottle with BOD nutrient mixture and aerated drinking water

3. Dilute the samples according to the dilution table

4. Fill two oxygen reaction bottles with diluted sample until they overflow and ensure they are free of air bubbles

5. Fill two oxygen reaction bottles with dilution water until they overflow and ensure they are free of air bubbles

6. Perform an oxygen concentration determination with one each of the bottles from points 4 and 5

7. Incubate the other bottles for n days at 20°C.

8. After the incubation, determine the final oxygen concentration in the reaction bottles.

9. Calculate the BOD value according to the given equation of the measured oxygen concentrations and the dilution ratio.

Components of the measuring system for determining the biochemical oxygen demand using the BOD cuvette test

The 00687 BOD cuvette test is used to determine the oxygen concentration. It contains all the reagents required for the color development reaction and the small glass beads that are used for mixing. The glass cuvettes are provided with a barcode to simplify the actual photometric determination.

The BOD nutrient mixture is a so-called lyophilisate. Lyophilisate means that it was freeze-dried for conservation. It contains the nutrients that are necessary for the bacteria. Moreover, it contains the nitrification inhibitor, i.e. allyl thiourea (ATU), in order to suppress the nitrification process. Thus, any additional ATU additive is superfluous.

The oxygen reaction bottles fulfill two tasks. On the one hand, they are used for the color development reaction and, on the other hand, they are the sample vessels using during incubation. In the case of the BOD measurement, an essential aspect is the bubble-free filling of the vessels for incubation as well as for the reaction. In order to ensure this, the stoppers are beveled.

The photometer is the actual measuring instrument. Through coded cuvettes and a special optic, it only remains to insert the cuvettes into the photometer and the result of the oxygen concentration measurement is obtained immediately. Changing the filter, method or factor inputs are no longer necessary in the new instruments.

For incubators and pipettes, measuring flasks, etc. the points already mentioned in the previous chapters apply. At this point, please refer to them for information. Nevertheless, please note again that incubation must be in the dark for vessels made of white glass.

Measurement

Dilution water

The biochemical oxygen demand is reduced so far by dilution that the oxygen that is present in the diluted solution is sufficient. The following dilutions are recommended for the BOD cuvette test:

BOD [mg/L]	12-50	50-100	100-500	500-1000	1000-3000
Sample + nutrient solution	1+9	1+19	1+99	1+199	1+499
Dilution factor	10	20	100	200	500

The seeded nutrient solution is produced in the following way:

20 mL settled wastewater that ideally comes from the influent to the biological purification stage of the plant that treats the wastewater is used as the seed material. The background to this is again the adapted biology of this sample. If this is not possible, domestic wastewater should be used.

The total contents of the small bottle with the nutrient mixture are dissolved in 1L chlorine-free drinking water. In order to achieve oxygen saturation, the drinking water is left to stand beforehand in an open beaker and stirred with a glass bar until approx. 20°C is reached. Afterwards, the solution can be assumed to be saturated with oxygen (approx. 9 mg/L oxygen). The nutrient mixture refers to a so-called lyophilisate. Lyophilisate means that the corresponding substances are freeze-dried for conservation. It then produces the dilution water and contains all the nutrients and mineral nutrients necessary for the bacteria.

Afterwards, the 20 mL seed are diluted to 1L with the dilution water and the required seeded dilution water is obtained which is used to produce the dilutions according to the table shown above.

Photometric measurement and incubation

The following sequence of operations is absolutely identical for the blank samples and the diluted samples for both the initial measurements and the final measurements. In the final measurements, the color development reagents are added only after incubation.
The first step is the addition of 1-2 glass beads to the empty reaction bottles. The glass beads have the task of mixing the sample and the reagents that are added later. Glass beads are necessary because no air bubbles must be present in the vessels. Shaking would otherwise change the oxygen content in the measurement sample to be determined. Without any air, shaking does not cause any mixing. The glass beads then ensure thorough mixing in the completely full vessels when they are shaken.
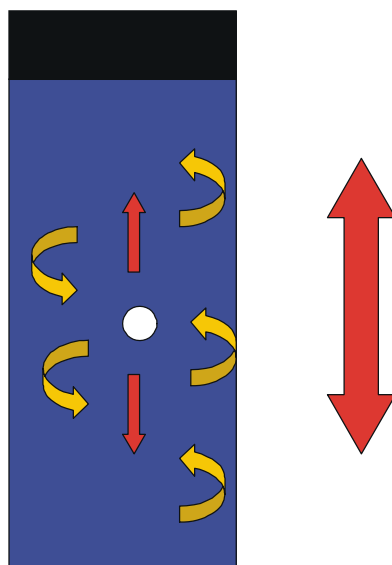
After the glass beads have been added to the reaction bottles, the diluted sample or the blank solution is added. The beveled stopper ensures that when the bottle is filled, it remains completely free of air bubbles.
The preparations that are intended for the final concentration determinations are placed in the incubator.
The other reaction bottles are opened again and 5 drops of the reagent BOD1-K and 10 drops of the reagent BOD-2K are added to each. Afterwards, they are closed again (free of air bubbles) and shaken.
(The reagents should be added as soon as possible after opening the bottles as, otherwise, the measurement can be falsified by oxygen from the air. When the reagents are added, some of the solution logically overflows when the vessel is closed again. For this reason, the surface under it should ideally not be a desk covered with important papers.)
Finally, 10 drops of the BOD reagent BOD-3K are added to each bottle which is closed again free of air bubbles and shaken.
Afterwards, the sample is immediately poured into the round cuvette that has been selected for the measurement and the oxygen concentration value is determined using the photometer. This really must be performed immediately because the dye that is

formed is stable for only a short period of time and, if the waiting time is too long, it would lead to a result that is too low. The glass beads may be allowed to fall to the bottom of the measuring cuvette as they lie below the beam of light in the photometer and do not affect the determination.

After 5 or 7 days (please, also note the time of day! If the test is started in the evening, the five days are only over in the evening and not already on the morning of the fifth day!), the photometric determination of the final oxygen concentrations can be performed. Open the reaction bottles, add BOD 1K, and so on.
As a result, all four values that are required for the calculation are now known.

$$BOD_n = \left[(c_1 - c_2) - (c_3 - c_4)\right] \cdot \frac{V_t}{V_e} = (A - B) \cdot \textit{dilution factor}$$

where:
c_1 Measured value [mg/L] of the measurement sample before incubation
c_2 Measured value [mg/L] of the measurement sample after n days
c_3 Measured value [mg/L] of the blank sample before incubation
c_4 Measured value [mg/L] of the blank sample after n days
V_e Volume of sample [mL] that was used for the production of the analysis solution in question
V_t Total volume [mL] of this analysis solution

or
A $=(c_1 - c_2)$ not corrected BOD_n of the measurement sample [mg/L]
B $=(c_3 - c_4)$ BOD_n of the blank sample [mg/L]

Repeat determinations are recommended. In this case, the average values of A and B must always be used for the calculation of the BOD_n of the original sample.

Note: The equation shown above differs from the equation for the calculation of the dilution BODs (DIN EN 1899-1 or Standard Methods 5210 B) in the omission of the factor $(V_t - V_e)/V_t$ in the portion of the blank sample. Within the framework of the possible accuracy of measurement, this simplification is acceptable and absolutely practical. According to the selected dilution, the factor can only adopt values between 0.9 and 0.998.

Appendix

List of the chemicals and solutions used in the Standard Methods and the Euronorm regulations

(A)   *Distilled water (Standard Methods 5210 D):*
Use only high-quality water distilled from a block tin or all-glass still. Deionized water may be used but often contains high bacterial counts. The water must contain less than 0.01 mg heavy metals/L and be free of chlorine, chloramines, caustic alkalinity, organic material, or acids. Make all reagents with this water. When other waters are required for special-purpose testing, state clearly their source and quality characteristics.

Respirometric method (*Standard Methods 5210 D*)

(R1)   *Phosphate buffer solution (1,5 molar):*
Dissolve 207 g sodium dihydrogen phosphate, $NaH_2PO_4 \cdot H_2O$, in water, Neutralize to pH 7.2 with 6N KOH (R6) and dilute to 1l.

(R2)   *Ammonium chloride solution (0,71 molar):*
Dissolve 38,2 g ammonium chloride, NH_4Cl, in water, Neutralize to pH 7.0 with KOH. Dilute to 1 L. 1 mL = 10 mg N.

(R3)   *Calcium chloride solution (0,25 molar):*
Dissolve 27,7 g Calcium chloride, $CaCl_2$, in water and dilute to 1 L; 1 mL = 10 mg Ca.

(R4)   *Magnesium sulfate solution (0,41 molar):*
Dissolve 101 g Magnesium sulfate, $MgSO_4 \cdot 7H_2O$, in water and dilute to 1 L; 1 ml = 1.0 mg Mg.

(R5)   *Ferric chloride solution (0,018 molar):*
Dissolve 4,84 g $FeCl_3 \cdot 6H_2O$ in water and dilute to 1 L. 1 mL = 1,0 mg Fe.

(R6)   *Potassium hydroxide solution (6 molar):*
Dissolve 336 g KOH in about 700 mL water and dilute to 1 L. Caution: Add KOH to water slowly and use constant mixing to prevent excessive heat buildup. Alternately, use commercial solutions containing 30-50% KOH by weight.

(R7)   *Acid solutions (1 molar):*
Add 28 mL conc. H_2SO_4 or 83 mL conc HCl to about 700 mL water. Dilute to 1L.

(R8)   *Alkali solution (1 molar):*
Add 40 g NaOH to 700 mL water and dilute to 1 L.

(R9)   *Nitrification inhibitor:*
Use TCMP (2-chloro-6-(trichlormethyl)pyridine) p.a. or a comparable substance if the inhibition of nitrification is required. While doing so, add 10 mg TCMP/L to the measurement solution.
Allyl thiourea ATU can also be used as the nitrification inhibitor. Here, a dosage of 5mg per liter of measurement solution is required.
If the ready-to-use WTW NTH600 solution is used, a dosage of 20 drops per liter of sample is required as NTH600 has a concentration of 5 g/L.

(R10) *Glucose-glutamic acid solution:*
Dry reagent-grade glucose and reagent-grade glutamic acid at 103°C for 1h. Add 15.0 g glucose and 15.0 g glutamic acid to distilled water and dilute to 1L. Neutralize to pH 7.0 using 6N potassium hydroxide (R6). This solution may be stored for up to 1 week at 4°C.

(R11) **_Sodium sulfite solution:_**
Dissolve 1,575 g sodium sulfite $Na_2SO_3 \cdot$ in about 800 mL water. Dilute to 1 L. The solution is not stable; prepare daily or as needed.

(R12) **_Trace element solution:_**
Dissolve 40 mg $MnSO_4 x \cdot 4H_2O$, 57 mg H_3BO_3, 43 mg $ZnSO_4 \cdot 7H_2O$, 35 mg $(NH_4)_6Mo_7O_{24}$, 100 mg Fe-Chelat ($FeCl_3$-EDTA) in about 800 mL water. Diute to 1 L. Sterilize at 120 °C and 200 kPa (2atm) pressure for 20 min.

(R13) **_Yeast extract solution:_**
Add 15 mg laboratory- or pharmaceutical-grade brewer's yeast extract to 100 ml water. Make this solution fresh immediately before each test in which it is used.

(R14) **_Nutrient solution:_**
Add

2,5	mL	phosphate buffer solution (R1)
0,65	mL	ammonium chloride solution (R2)
1,0mL		calcium chloride solution (R3)
0,22	mL	magnesium sulfate solution (R4)
0,1mL		ferric chloride solution (R5)
1,0mL		trace element solution (R12)
1,0mL		yeast extract solution (R13)

to about in 900 mL water. Dilute to 1 L. This nutrient solution and those of R12 and R13 above are specially formulated for use with the OECD method. (Note: A 10:1 concentrated nutrient solution can be made and diluted accordingly.

(R15) **_Seed source according to Standard Methods – 5210 B BOD$_5$ Test – 19th edition – 1995_**
Some samples do not contain a sufficient microbial population (for example, some untreated industrial wastes, or wastes with extreme pH values). For such wastes seed the dilution water by adding a population of microorganisms. The preferred seed is effluent from the biological treatment system processing the waste. Where this is not available, use supernatant from domestic wastewater after settling at room temperature for at least 1 h but not longer than 36 h. When effluent from a biological treatment process is used, inhibition of nitrification is recommended. Some samples may contain materials not degraded at normal rates by the microorganisms in settled wastewater. Seed such samples with adapted microbial population obtained from undisinfected effluent of a biological process treating the waste. In the absence of such a facility, obtain seed from the receiving water below (preferably 3 to 8 km) the point of discharge. When such seed sources also are not available, develop an adapted seed in the laboratory by continuously aerating a sample of settled domestic wastewater and adding small daily increments of waste. Optionally use soil suspension or activated sludge, or a commercial seed preparation to obtain the initial microbial population. Determine the existence of a satisfactory population by testing the performance of the seed in BOD tests on the sample. BOD values that increase with time of adaptation to a steady high value indicate successful seed adaptation.

Dilution method (DIN EN 1899-1, ISO 5815, Standard Methods 5210 B) (not authorized translation)

(V1)   ***Phosphate-buffer solution pH 7.2***
Dissolve 8,5 g potassium dihydrogen phosphate (KH_2PO_4), 21,75 g dipotassium hydrogen phosphate (K_2HPO_4), 33,4 g disodium hydrogen phosphate heptahydrate ($Na_2HPO_4 \cdot 7\,H_2O$) and 1,7 g ammonium chloride (NH_4Cl) in about 500 mL water. Dilute to 1 L and mix.

(V2)   ***Magnesium sulfate, solution, 22,5 g/L***
Dissolve 22,5 g magnesium sulfate heptahydrate ($MgSO_4 \cdot 7\,H_2O$) in water. Dilute to 1 L and mix.

(V3)   ***Calcium chloride, solution 27,5 g/L***
Dissolve 27,5 g anhydrous calcium chloride ($CaCl_2$)(or an equivalent quantity if the hydrate is used) in water. Dilute to 1 L and mix.

(V4)   ***Ferric-chloride, solution 0,25 g/L***
Dissolve 0,25 g Ferric(III) chloride hexahydrate ($FeCl_3 \cdot 6\,H_2O$) in water. Dilute to 1 L and mix.

(V5)   ***Glucose-glutamic acid,*** *standard solution*
Dry water-free D-glucose ($C_6H_{12}O_6$) and L-glutamic acid ($C_5H_9NO_4$) at (105 ± 5)°C for 1 h. Weigh out (150 ± 1) mg of each substance, dissolve it in water, dilute to 1000 mL and mix. The theoretical oxygen demand of this solution is 307 mg/L oxygen (the empirical BOD_5 is 210 ± 40 mg/L). Make the solution immediately before use and discard any remaining solution at the end of the working day. The solution can also be frozen in small quantities. The defrosted solution must be used immediately after it has been defrosted.

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