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# Microbial activity assessment based on hydrogen peroxide decomposition rates

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**Fast, easy, reliable method offers great potential for all aquaculture systems**



The new method described here is a fast, easy and reliable alternative that allows rapid (1–2 hours) and simple quantification of microbial activity in fresh- and saltwater samples from aquaculture systems.

There is a widespread need for cultivation-free methods to quantify the viability of microbial communities in aquatic environments. This demand also exists within the aquaculture industry, where rapid and reliable methods for measuring bacterial activity and bioavailable organic matter in water are increasingly required.

Microbial water quality assessment has the potential to provide insights into the temporal and spatial dynamics of bacterial communities within aquaculture systems as a supplement to general chemical water quality parameters. This is of particular relevance to recirculating aquaculture systems (RAS) that are characterized by a continuous input of bioavailable substrates, high nutrient levels and long retention times all favoring heterotrophic bacterial growth.

The use of methods for assessing the bacterial and the broader microbial activity in water may contribute to gain additional knowledge of potential effects and interaction of various factors (i.e. feed composition, feed loading, hydraulics and mechanical, biological and chemical treatments) on bacteriology and growth potential in RAS. Easy and reliable methods are therefore crucial to the ongoing development of RAS because they can provide data for baseline conditions as well as detection of sudden unforeseen deviations.

Determination of biological oxygen demand over five days ( $BOD_5$ ) is a common method to quantify the bioavailable organic matter in aquaculture water. The method is informative on biodegradable organic matter but is inexpedient with respect to time. Traditional methods, such as plate counting to obtain colonies forming units (CFU) are sometimes used to predict changes in relative numbers of bacteria in aquaculture water samples.

This article – adapted and summarized from the **original publication** (<https://doi.org/10.1016/j.aquaeng.2019.01.001>) in *Aquacultural Engineering* – describes our proposed, rapid, simple, inexpensive and reproducible method that reflects the microbial enzymatic activity of both free living and particle associated bacteria, as well as potential contributions from other

microbiota. The underlying principle takes advantage of  $\text{H}_2\text{O}_2$  decomposition (also referred to as degradation, elimination, reduction or decay) which is primarily a biological process and hence related to the microbial composition of the water.

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Water quality monitoring and control is important for successful aquaculture production. These pictures show a Danish flow through pond (short retention time and limited accumulation of organic matter) with rainbow trout.

## Study setup

The method of this assay is based on adding a well-defined quantity of  $\text{H}_2\text{O}_2$  to a raw water sample and quantifying the decomposition of  $\text{H}_2\text{O}_2$  over time under controlled conditions. Under constant conditions (nominal  $\text{H}_2\text{O}_2$  concentration, temperature and mixing), the decomposition rate reflects amounts of total enzymatic activity of bacteria and any eukaryotic microbiota present (planktonic and particle bound).

For all batch experiments, the  $\text{H}_2\text{O}_2$  and chemical oxygen demand (COD) concentrations as well as the 5-day biological oxygen demand ( $\text{BOD}_5$ ) were measured in at least duplicates and the average values were used. Water volumes,  $\text{H}_2\text{O}_2$  concentration and sampling frequency are modifiable as long as consistent mixing and temperature control are provided. An applied fixation reagent terminates the  $\text{H}_2\text{O}_2$  decomposition and forms a stable color complex allowing flexible sampling and subsequent spectrophotometric analysis.

The water samples used were all collected from a pilot scale freshwater recirculating aquaculture system (RAS) operated with rainbow trout at different densities and feed loadings. To investigate the effects of filtration on  $\text{H}_2\text{O}_2$  decomposition kinetics, water was collected from a RAS operated at constant conditions for a period of four months. To compare  $\text{H}_2\text{O}_2$  decomposition rate constants with



BOD<sub>5</sub> analysis, water samples were collected from six different RAS over a three-week period). Finally, H<sub>2</sub>O<sub>2</sub> decomposition rate constants were compared with micro particle numbers based on water samples from 12 different RAS operated at constant conditions.

For additional information on general description of the study; the procedure for H<sub>2</sub>O<sub>2</sub> decomposition assay; reagents and chemical methods used; theory and calculations; description of RAS systems for samples; and statistical analyses, please consult the original publication or the first author.



Water samples used in this study were collected from a variety of pilot scale RAS reflecting water quality conditions from commercial trout production.

## Results and discussion

A linear standard curve based on H<sub>2</sub>O<sub>2</sub> standard solutions from 0.5 to 25 mg H<sub>2</sub>O<sub>2</sub>/L was used to measure H<sub>2</sub>O<sub>2</sub> in water samples spiked with H<sub>2</sub>O<sub>2</sub>. The H<sub>2</sub>O<sub>2</sub> concentration was then calculated as  $[H_2O_2] = (Abs_{432} - 0.0022)/0.0132$  with that particular reagent. The level of detection of the applied method was 0.16 mg/L H<sub>2</sub>O<sub>2</sub>, corresponding to  $\leq 0.002$  Abs units, and H<sub>2</sub>O<sub>2</sub> concentrations were adjusted for background absorbance and used to calculate decomposition rate constants for H<sub>2</sub>O<sub>2</sub>.

Regarding the effects of filtration, the H<sub>2</sub>O<sub>2</sub> decomposition rate constants derived from raw and pre-filtered RAS water confirmed that the majority of H<sub>2</sub>O<sub>2</sub> decomposition was related to particle associated bacterial activity. The rate constant in 0.2  $\mu m$  sterile filtered RAS water at 22 degrees-C was 0.038/hours with a corresponding H<sub>2</sub>O<sub>2</sub> half-life of  $T_{1/2} = 18.2$  hours. Planktonic bacteria and small aggregates in the size range from 0.2 to 1.6  $\mu m$  had a seven-fold higher decomposition rate constant with  $k = 0.263 \text{ h}^{-1}$  ( $T_{1/2} = 2.6$  hours), while the H<sub>2</sub>O<sub>2</sub> decomposition rate constant in unfiltered RAS water at 22 degrees-C was  $1.425 \text{ h}^{-1}$  ( $T_{1/2} = 0.49$  hours).

The COD content of the unfiltered RAS water samples was  $88.5 \pm 0.1 \text{ mg O}_2/\text{L}$ , while 1.6  $\mu m$  and 0.2  $\mu m$  filtration reduced the COD content to  $40.1 \pm 0.2 \text{ mg O}_2/\text{L}$  and  $35.9 \pm 0.2 \text{ mg O}_2/\text{L}$ , respectively. These values, along with accumulated nitrate and phosphate, indicates that RAS are nutrient rich environments with both dissolved and particulate organic matter favoring heterotrophic bacterial

growth as opposed to the oligotrophic conditions in some natural waters. The direct correlation between biodegradable organic matter and rate of  $\text{H}_2\text{O}_2$  decomposition was recently confirmed in RAS with different feed loadings, in RAS with acetate supplements and in a survey of seven commercial Danish model trout farms.

The abundance and distribution of planktonic bacteria versus particle associated bacteria and small eukaryotic microorganisms are likely to differ among individual RAS and over time. The exact causes, mechanisms and potential implications require further research. Site specific conditions and associated microbial composition may also reveal interesting enzymatic contributions from bacteria, algae and protozoa.

The  $\text{H}_2\text{O}_2$  decomposition rate constants were significantly affected by temperature in the unfiltered RAS water sample (Fig. 1). With values of  $0.979 \text{ h}^{-1}$  at 17 degrees-C and  $1.425 \text{ h}^{-1}$  at 22 degrees-C, a temperature coefficient of 1.078 (7.8 percent increase per degree-C) was calculated.

Fig. 1:  $\text{H}_2\text{O}_2$  decomposition rate constant (k) derived from freshwater RAS samples. Bars represent mean  $\pm$  std. dev. (n=3) based on spiking  $\text{H}_2\text{O}_2$  trials with unfiltered and pre-filtered ( $0.20 \mu\text{m}$  sterile filter and  $1.6 \mu\text{m}$  GFA filters) water samples at 17 and 22 degrees-C. Organic matter measured as COD was  $88.5 \pm 0.1 \text{ mg O}_2/\text{L}$  (unfiltered);  $40.1 \pm 0.2 \text{ mg O}_2/\text{L}$  ( $1.6 \mu\text{m}$ ) and  $35.9 \pm 0.2 \text{ mg O}_2/\text{L}$  ( $0.2 \mu\text{m}$ ). Different letters denote highly significant differences between groups ( $p < 0.01$ ). [Source: Aquacultural Engineering, 2019. Vol. 85:9-14].

We determined a highly significant positive correlation between  $\text{H}_2\text{O}_2$  decomposition rate constants and bioavailable organic matter measured as standardized oxygen consumption over five days ( $\text{BOD}_5$ ). The  $\text{H}_2\text{O}_2$  decomposition assay describes the actual microbial activity in a sample as opposed to an indirect measure of organic matter for potential bacterial growth provided by  $\text{BOD}_5$  and COD.

The  $\text{H}_2\text{O}_2$  decomposition rate constants reached 3.7/hours at 15 degrees-C. As  $\text{BOD}_5$  reflects the amount of bioavailable dissolved and particulate organic matter, this parameter gives a more exact description of the bacterial growth potential than COD, which is nevertheless often used as a proxy due to its faster processing time. The  $\text{H}_2\text{O}_2$  decomposition assay described here has potential to become a supplementary or alternative method to  $\text{BOD}_5$  and COD measurements since it correlates well with both parameters.

The  $\text{H}_2\text{O}_2$  assay has a high reproducibility and allows fast measurements in both fresh and saline water samples where dilutions are often needed for  $\text{BOD}_5$  and COD. The  $\text{H}_2\text{O}_2$  decomposition rate constant was also strongly correlated with the quantity of micro particles in RAS water samples. This means that our simple methods can be used as a proxy for both  $\text{BOD}_5$  and micro particle concentration.

The  $\text{H}_2\text{O}_2$  decomposition assay has potential important applications. Bacteria in the water are suspended, aggregated and attached to particles, and they potentially challenge the performance of fish and RAS. The factors affecting microbial composition and dynamic, their virulence and associated causal relationships are likely to become a research area of high importance for the aquaculture industry in the near future.

Fig. 2: Theoretical/modeled decomposition scenarios of  $\text{H}_2\text{O}_2$  calculated based on nominal  $\text{H}_2\text{O}_2$  concentration of 10 mg/L and various decomposition rates. The figure makes it possible to determine the k-value based on controlled  $\text{H}_2\text{O}_2$  addition and a time specific measurement of the residual  $\text{H}_2\text{O}_2$ . [Source: Aquacultural Engineering, 2019. Vol. 85:9-14].

## Perspectives

The assay presented here provides direct information on microbial activity in the water with a short-duration measurement. Fig. 2 conceptualizes how to apply the method in a practical way where an approximate rate constant is assessed based on time-flexible single measurements. In oligotrophic water, or water from advanced RAS with UV, ozone disinfection and efficient solids removal, increasing exposure time will improve resolution of the assay.

At the other end of the spectrum, the  $\text{H}_2\text{O}_2$  assay can be made with shorter duration and increased  $\text{H}_2\text{O}_2$  addition when assessing microbial rich eutrophic water from natural water bodies or open aquaculture pond systems with high feed loading and long water retention time.

As a fast and new tool to describe microbial activity,  $\text{H}_2\text{O}_2$  decomposition assay are applicable for:

- Evaluating microbial activity in an aquaculture facility on a regular basis. This will give new information, increase system understanding and make it possible to establish baseline conditions and detect deviations.
- Quantifying and evaluating the effect of a given treatment component (foam fractionator, mechanical and biological filters etc.) and changes in operational practices (disinfection, new feed, altered fish densities, hydraulic, etc.).
- Improving planning and optimizing water treatment and sanitation (disinfection demand).
- Providing measurements of microbial activity and dynamics in field and laboratory trials.
- Replacing COD (and  $\text{BOD}_5$ ) measurements particularly for saline samples.

The assay could also be modified to quantify bacterial activity in biofilms or on system level. Modifications of the method might include simplifications (strip sticks), automation (e.g. using an online  $\text{H}_2\text{O}_2$  sensor), use of other reagents, or use of plate reader assays to increase throughput.

In conclusion, the study has demonstrated that hydrogen peroxide decomposition is a rapid, efficacious and feasible indicator of microbial activity in water samples. The  $\text{H}_2\text{O}_2$  assay has several potential applications in aquaculture worldwide from pond farming to intensive RAS as it provides new knowledge of the microbial water quality conditions in a fast, easy, competitive and reliable way. The new method we describe here can most likely also be applied in other areas, for example waste water quality determination.

*Further details can be found in the original publication: Pedersen, L.F., Rojas-Tirado, P.A., Arvin, E., & Pedersen, P.B. (2019). Assessment of microbial activity in water based on hydrogen peroxide decomposition rates. Aquacultural Engineering, 85, 9-14.*

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