Nanoparticle-based measurements of pH and O₂ dynamics in the rhizosphere of Zostera marina L.: Effects of temperature elevation and light-dark transitions

Kasper Elgetti Brodersen¹,a,*, Klaus Koren²,a, Mads Lichtenberg²,

Michael Kühl¹,²,*

¹Plant Functional Biology and Climate Change Cluster,
University of Technology, Sydney
15 Broadway, Ultimo, Sydney, NSW 2007, Australia

²Marine Biological Section, Department of Biology,
University of Copenhagen
Strandpromenaden 5, DK-3000 Helsingør, Denmark

*a These authors contributed equally to this work.
* Corresponding author, e-mail: mkuhl@bio.ku.dk and phone: +45 4047 6304
SUPPORTING FIGURES AND NOTES

Figure S1.

Luminescence spectra of the optical pH nanosensors in alkaline (pH 10; green) and acidic (pH 3; orange) solutions, showing a marked drop in luminescence in the yellow-orange-red wavelength interval (∼550-675 nm) combined with an increase in the violet-blue-green wavelength interval (∼430-530 nm) under acidic conditions. The nanoparticles were excited by a 405 nm LED and the spectra were recorded with a fiber-optic spectrometer (QE65000; oceanoptics.com).
Figure S2

Calibration of pH nanosensor luminescence. Ratio images, i.e., the ratio of red and blue channels extracted from the recorded RGB image, were quantified in small transparent glass vials with pH nanoparticle-containing agar buffered to defined pH levels spanning pH 4-10.

The pH sensor nanoparticles were calibrated as follows. A solution of ~0.5% (w/w) agar-NaCl water (with a salinity of 34) was poured into small glass vials (3 mL volume). To adjust the pH value, 300 µL of a 100 mM buffer solution (citrate, phosphate or TRIS) were added. At a temperature of ~40°C, a small volume of the pH sensitive nanoparticles was added to a final concentration as used in the artificial sediment. After a short mixing step the agar was left to solidify. The glass vials were kept at constant temperature (16°C or 24°C) in a thermostated water bath and were then imaged with the ratiometric camera system using identical settings as for the seagrass sample. The ratio (red/blue channel) images were then correlated to the known pH values.

<table>
<thead>
<tr>
<th>pH</th>
<th>Ratio (red/blue channel)</th>
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<tbody>
<tr>
<td>4.0</td>
<td>2.47 2.58 3.25 4.50 5.16 5.27 5.31</td>
</tr>
<tr>
<td>5.0</td>
<td>1.59 1.63 1.87 2.67 3.99 4.53 4.82</td>
</tr>
<tr>
<td>6.0</td>
<td>7.0</td>
</tr>
</tbody>
</table>
Figure S3. Calibration curves for optical pH nanoparticle-based sensors at the two experimental temperatures 16 and 24 °C. Mean ratio values were fitted with a sigmoidal function ($r^2 = 0.99$ and 0.97, respectively). Error bars are ± SD (n=3).
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**Figure S4.**

Vertical pH microprofiles in the bulk, artificial sediment containing pH sensitive nanoparticles measured with both a calibrated pH microelectrode (pH-50, tip diameter of ~50 µm; Unisense A/S, Aarhus, Denmark; Kühl & Revsbech, 2001) and the optical nanoparticle-based pH sensors. There was a high resemblance between pH microprofiles (and thereby the sediment pH levels) determined with the two different sensor types. The pH levels of the bulk, artificial sediment dropped rather rapidly in the first 0-5 mm depth from ~pH 7.5 at the seawater/sediment interface to ~pH 6 at the approximate position of the below-ground biomass, thus mimicking chemical settings in natural sediment (Burdige and Zimmerman, 2002; Stahl et al. 2006; Zhu et al. 2006) (further information about the casting procedure of the reduced, artificial sediment is provided in Brodersen et al. (2014)).

*pH microelectrode measurements.* The pH microelectrode was mounted on a micromanipulator (Unisense A/S, Denmark) and used in combination with a reference electrode (tip diameter of ~5 mm; Unisense A/S) immersed in the supporting water reservoir; both connected to a pH/mV-Meter (Unisense A/S). Before measurements commenced, the pH microelectrode was linearly calibrated from sensor readings in three pre-known pH buffers (pH 4, 7 and 9; linearly responding over the pH calibration range with a signal to pH ratio of 51 mV/pH unit) at experimental temperature and salinity. The microelectrode measurements were performed in the custom-made narrow split-flow chamber (Fig. 1), in the same area as the extracted vertical pH microprofiles obtained via the pH sensitive nanosensors, by manually handling the micromanipulator (increments of 1 mm). The artificial sediment surface was determined by manually moving the microsensor towards the seawater/sediment interface, while observing the microsensor tip and the sediment surface through a magnifying glass, as well as from signal readings.
Figure S4. pH microprofiles measured in the bulk, artificial sediment containing pH sensitive nanoparticles with a pH microelectrode (red symbols; mean ± SD; n=3) and with the optical nanoparticle-based sensors (black line). Y = 0 indicates the artificial sediment surface.
Figure S5. Calibration curves of optical O$_2$ nanosensors measured at the two experimental temperatures (Blue = 16°C; Red = 24°C; mean red/green ratio values were fitted with an exponential decay function, $r^2 = 0.99$). The optical O$_2$ nanoparticle-based sensors incorporated in the artificial, transparent sediment were calibrated as described in Koren et al. (2015).
When working with optical (luminescence-based) sensors several factors can lead to artefacts and consequently misinterpretation of the results. The following paragraph intends to create awareness of potential problems and gives direction for possible solutions.

### Intensity, ratiometric and lifetime based imaging:

In general, three different types of luminescence imaging are used for readout of optical chemical sensors. The most error prone is simple luminescence intensity-based imaging, where the intensity of a pixel or region is correlated to the analyte concentration. This approach is affected by numerous potential artefacts such as fluctuations in the illumination source, uneven illumination and/or distribution of the luminescent indicator, interference from background illumination and bleaching of the sensor material. In order to overcome some of these potential artefacts, a ratiometric imaging approach (where the ratio between the luminescence intensity of an analyte-sensitive indicator dye and the luminescence intensity of an inert reference dye is correlated to the analyte concentration) can be used to correct for uneven illumination or sensor distribution, and fluctuations in the illumination source. In terms of bleaching effects, a similar bleaching rate of the indicator and reference dyes is favourable; if one of them bleaches more easily than the other, this will lead to erroneous analyte concentrations. Background light and autofluorescence remains a problem in ratiometric imaging. Ratiometric imaging can be realized with relatively simple camera systems, like the SLR camera system used in this study (Larsen et al. 2011). Lifetime-based imaging, where the analyte-dependent change in the indicator luminescence decay time is monitored, is a very good and reliable alternative method but involves complicated and expensive instrumentation. Further details on this topic can be found in Meier et al. (2013).

### Planar optodes vs. nanoparticle-stained artificial sediments

As this study utilizes nanoparticle-based sensors incorporated into transparent, artificial sediment we want to briefly discuss the benefits, but also potential artefacts, of this novel methodology in particular in comparison to the more commonly used planar sensor optode methods. Further details about the planar sensor optode method can be found in Santner et al. (2015). In brief, the use of planar optodes enables chemical imaging in the plane of an optical sensor foil. Analysis of complex structures like the below-ground tissue of seagrasses requires a close proximity of the planar optode to the tissue surface. This is often difficult, if not impossible, to achieve and can limit the part of the belowground tissue that can actually be analysed. In contrast, the nanoparticle method applied in this study enables simultaneous imaging of the entire below-ground tissue of seagrasses on a whole rhizosphere level (Koren et al. 2015).

An advantage of planar optodes is that an optical isolation can be applied on top of the sensor film. This protects the sensor from external light and protects the structure to be analyzed from the sensor excitation light (Glud et al. (1996)). The latter is particularly important when high energy light (e.g. UV light) is used for excitation as this can easily cause background fluorescence from biological samples. An optical isolation layer can obviously not be applied.
in case of the nanoparticle stained transparent, artificial sediment. This means that things like background fluorescence from the sample have to been taken into account when interpreting the images.

Avoiding artefacts when working with intensity or ratiometric based imaging

In general the following rules apply when trying to avoid potential artefacts

- Use an excitation light that will not cause autofluorescence in the biological sample.
  - If this is not possible, as the indicator requires a certain excitation wavelength, try to image the sample without the sensor particles to see how high the background is and subtract this if needed in the subsequent image analysis.

- Be aware of color-dependent scattering
  - Use as thin a layer of artificial sediment as possible, to reduce the light path, and use color-corrected optics

- Avoid background light
  - If not possible, e.g. if darkening of the room is not entirely possible; take an image with the excitation light source off. This “dark” picture can then be subtracted from the images with the excitation light on. Nevertheless, it is advisable to get the surroundings as dark as possible.

- Bleaching: In case long-term light exposure is planned, it is recommended to test photostability prior to the measurements and account for potential photobleaching.

- Calibration: It is recommended to calibrate the sensor with the exact same conditions/settings as planned for the later measurements.

In the present study, all of the above-mentioned precautions were followed/secured during imaging. As UV light had to be used for pH imaging (e.g. Larsen et al. 2011), a few potential artefacts associated with the high energy excitation light were observed and are discussed below. All other above mentioned sources of potential artefacts could be excluded from the images.

Artefacts seen in the pH images

Artefacts are often easier to discover in the raw images of the different colour channels of the recorded RGB images. As seen in Figure S6, the blue colour channel appears to have a homogeneous intensity distribution (A), while the red channel shows some spots of high light (B). Especially in biological samples, this can e.g. be due to background fluorescence induced by the sensor excitation light. As the ratio of the two colour channels is used to calculate the pH image, such artefacts results in locally false pH readings. For example, Figure S6 depicts 4 small areas with very high red luminescence (white arrows, B) associated with leaves and prophyllums, that are probably partly covered by epiphytes, where the blue excitation light induced chlorophyll-derived red background luminescence. In contrast to
other regions that show dynamics in response to altered environmental conditions (such as at the rhizome; Fig. S6, A), the 4 high red luminescence areas remain unchanged.

All images were therefore interpreted with caution. Potential artefacts were thoughtfully analysed, as demonstrated in Figure S6. Signals from regions potentially affected by artefacts (such as epiphyte-derived red background luminescence) were excluded from further analysis and marked on presented images by arrows, to ensure sufficient precaution was taken into account when interpreting the images. This is, as previously mentioned, a minor limitation of current pH imaging, as high energy excitation light has to be used when exciting the pH sensitive indicator/reference dyes.

**Figure S6.** Visualization of potential artefacts in the obtained pH images (images are from the 16°C treatment). The blue and red channel images are obtained by splitting the original RGB picture into its respective colour channels. The blue channel image (A) appears quite homogeneous in terms of intensity, while the red channel image (B) shows several high intensity regions. When merging the two channels (C) it can be seen that most of the picture appears in a homogeneous pink colour, while the hotspots in the red picture remain. This subsequently leads to very high apparent pH values at those spots as the ratio of red and blue channel leads to the final pH image (D). In contrast to other regions (e.g. low pH hotspot at the rhizome; A) those spots do not change over time and in response to the altered light levels and/or temperature. An additional artefact is presented by the region on top of the artificial sediment (e.g. square in the pH image; D). In this region the measured intensities are not due to the optical nanoparticle based sensors and only represent noise such as scattered light, wherefore this region has been excluded.
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REFERENCE LIST


