

# A Protocol for Routine Monitoring for *Karenia brevis* using Gyroxanthin-Based Detection Methods

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## SUMMARY

This protocol has been developed for use in routine monitoring for the toxic dinoflagellate *Karenia brevis*. The method is based on detection of the biomarker pigment gyroxanthin-diester using high performance liquid chromatography (HPLC). Previous research has shown that gyroxanthin-diester is a reliable indicator of *Karenia brevis* abundance in coastal waters (e.g. Millie et al 1995, 1997, Kirkpatrick et al 2000, Ómólfisdóttir et al 2003, Richardson & Pinckney, 2004). For details of experiments that contributed to the development of this protocol see Richardson & Pinckney (2004).

## PROTOCOL

### 1. Sample Collection and Storage

At each monitoring station, samples should be collected from the surface and from deeper in the water column (as desired, and as logistics permit) using standard sampling devices (integrated sampler or Niskin bottle). Collect at least 4 liters of water from each location/depth. This volume should provide more than enough sample to provide replicate samples for detection *Karenia brevis* at concentrations near the FDA-mandated limit for closure of shellfish beds (5 cells/ml) for *Karenia brevis*, based on current HPLC detection limits (see below). Samples should be pre-filtered with a 200 µm Nitex screen (or comparable) to remove large grazers. All samples should be stored in opaque bottles in coolers that contain just enough ice to keep the sample at ambient temperature (or slightly cooler) but samples, at this stage, should not be allowed to freeze. Always protect samples from bright light. Samples may be stored for up to 8 hours with no detrimental effects, however efforts should be made to filter samples as soon as possible after collection.

### 2. Filtration of Samples

For filtration of samples, use a standard filtration set-up of manifold, sample cups, and vacuum pump. Filtrations should be done in a room with only dim light. A red light may be used for illumination. The following filters (47 mm size) may be used for filtration: GF/C, 934-AH, or GF/A. All gave acceptably good retention of *Karenia brevis* cells with relatively short filtration times (Richardson and Pinckney, in review). Pressure on the vacuum pump should be monitored carefully and always should stay below 200 mmHg. Detection of low (< 5 cells/ml) concentrations of *Karenia brevis* requires the filtration of > 500 ml of sample. The greater the biomass of *Karenia brevis* on the filters, the better the measurement of gyroxanthin-diester, thus researchers should try to filter the maximum amount of material possible until filtration times become unacceptably long (>10 minutes per sample). Note that filtration times will vary widely depending on the concentration and size distribution of the bulk phytoplankton community and suspended sediment in the sample.

Filter a minimum of three replicates for each depth or location sampled. We recommend quadruplicate samples if resources and patience permit. After each sample is filtered, rinse the filter well with filtered sea water, remove from the filtration apparatus, fold the filter in quarters and then blot it gently but thoroughly between a piece of folded paper towel to remove excess water from the filter. The folded filter should be placed into a small (2 ml) plastic Eppendorf centrifuge tube (labeled) or cryotube and should be wrapped in aluminum foil and immediately put into liquid nitrogen or into a -80°C freezer. Samples can remain in the -80°C freezer for up to one year with no adverse effects. Samples flash-frozen in liquid nitrogen can also be stored in a -80°C freezer long term.

### 3. HPLC Determination of Gyroxanthin-diester

#### Analytical Instrumentation:

HPLC Capable of running a binary gradient (i.e. dual pumps or solvent mixer)  
Large volume injection loop (~500 ml)  
Spectrophotometric detector (440 nm fixed wavelength)  
Refrigerated autosampler (2°C) optional  
Column oven (capable of 40°C) optional  
Photodiode array spectrophotometer (350-700 nm) optional

#### HPLC Columns:

Guard column- monomeric reverse-phase C<sub>18</sub> column  
Recommendation: Rainin Microsorb, 0.46 x 1.5 cm, 3 mm packaging  
First analytical column- monomeric reverse-phase C<sub>18</sub> column  
Recommendation: Rainin Microsorb-MV, 0.46 x 10 cm, 3 mm packaging  
Second analytical column- polymeric reverse-phase C<sub>18</sub> column  
Recommendation: Vydac 201TP, 0.46 x 25 cm, 5 mm packaging  
Analytical columns are connected in series, with the monomeric column first and the polymeric column second.

#### Solvents and Reagents:

Methanol (HPLC grade)  
Acetone (HPLC grade)  
Ammonium acetate (analytical grade)  
Deionized water

#### Solvent System

Solvent A: 80% methanol 20% ammonium acetate (0.5 M adjusted to pH 7.2)  
Solvent B: 80% methanol 20% acetone  
Ion-pairing solution: 1.0 M ammonium acetate

#### a) Extraction

Under dim (or red) light, samples should be removed from the -80°C freezer and placed on ice. Remove frozen filter from each tube, one at a time, and cut the filter crosswise (length and width) into small pieces. This will enhance the absorption of solvent. Collect the chopped pieces of filter with small forceps and return them to the Eppendorf tube. Add 1 ml of 100% acetone to the tube, cap immediately, and place in a -20°C freezer. Repeat for as many samples as required. Samples may be sonicated (Fisher Sonic Dismembrator, model 300, with microtip) for 30 - 60 sec in an ice slurry to reduce heating if desired. Samples in acetone should remain in the -20°C freezer, in the dark, for at least 24h for extraction.

#### b) Chromatographic separation of pigments

Under dim room lights, remove extracts from the -20°C freezer one or a few at a time (to avoid warming). Samples are prepared for analysis by HPLC as follows. First, use a small syringe plunger to concentrate the pieces of filter in the bottom of the Eppendorf tube, and use a needle or other fine pipette to pull off the supernatant. Then, filter this supernatant through a 0.45 µm PTFE filter (Gelman Acrodisc). The extract (750 to 1000 µl) should then be dispensed into amber glass autosampler vials (1.8 ml), sealed with teflon-lined caps, and placed in the autosampler for HPLC analysis. Just prior to the HPLC run, an ion-pairing (IP) solution (1 M ammonium acetate) should be added to the vial in a ratio of 3 parts extract 1 part ammonium acetate. Prior work has shown that there is negligible pigment degradation within 12 h of adding the IP solution if the sample is placed in a refrigerated autosampler rack. However, the IP solution should not be added to the sample if the time until sample analysis is greater than 18 hours. Many HPLC systems have the capability for in-line mixing of reagents prior to the run and this is the preferred method for adding the IP solution.

Filtered extracts (375 µL) are injected into a Shimadzu HPLC equipped with a single monomeric and one polymeric reverse-phase C<sub>18</sub> column in series. This column configuration enhances the separation of photopigments and pigment

degradation products. Monomeric columns provide strong retention and high efficiency, while polymeric columns select for similar compounds with minor differences in molecular structure and shape (Van Heukelem et al. 1994; Jeffrey et al. 1997). A nonlinear binary gradient, adapted from Van Heukelem et al. (1994), is used for pigment separations (Pinckney et al. 1996). Solvent A consists of 80% methanol:20% ammonium acetate (0.5 M adjusted to pH 7.2) and solvent B is composed of 80% methanol:20% acetone. Our HPLC system consists of a Shimadzu SPD-M10av photodiode array (PDA) detector with accompanying Shimadzu components (SCL-10A controller; SIL-10A injector; and a CDO-10AS column oven). Pigment peaks should be identified by comparison of retention times and absorption spectra with pure standards of chlorophyll a, (Sigma Chemical Company) and gyroxanthin (DHI, see address and contact information below). The HPLC gradient programs are listed on page 6.

#### 4. Conversions of Pigment Concentrations to Cell Concentrations

The regulatory limit for closure of shellfish beds and beaches due to *Karenia brevis* is expressed in units of cells ml<sup>-1</sup> (5 cells ml<sup>-1</sup>). Thus, pigment concentrations must be converted to cells ml<sup>-1</sup> using a gyroxanthin cell<sup>-1</sup> conversion factor. Work by us and in other laboratories has shown that there is considerable geographic variability in gyroxanthin cell<sup>-1</sup> (Millie et al. 1995, Ómólfssdóttir et al. 2003, Higham et al. 2003, Richardson and Pinckney, in review). Thus, we recommend that a preliminary assessment of this conversion factor be done for the site of interest. Conversely, a relatively conservative value of 0.2 pg gyroxanthin cell<sup>-1</sup> may be used to convert pigment concentrations to cell concentrations.

Pigment standards for calibration may be obtained from

The International Agency for <sup>14</sup>C Determination  
DHI- Water and Environment  
Ager Allé 11  
DK-2970 Hørsholm  
Denmark  
<http://www.c14.vki.dk>  
c14@dhi.dk

#### References:

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## HPLC Gradient Programs:

	Time	% Solvent A	% Solvent B	Flow Rate (ml/min)
Startup Program	0.00	0	100	1.00
	5.00	100	0	0.80
	10.00	100	0	0.80
	Next			

	Time	% Solvent A	% Solvent B	Flow Rate (ml/min)
Gradient Program	0.00	100	0	0.80
	5.00	0	100	0.80
	10.00	0	100	1.00
	10.01	0	100	1.50
	20.00	0	100	1.50
	20.01	100	0	0.80
	22.00	100	0	0.80
	Next			

	Time	% Solvent A	% Solvent B	Flow Rate (ml/min)
Shutdown Program	0.00	100	0	0.80
	5.00	0	100	1.00
	10.00	0	100	1.00
Stop				

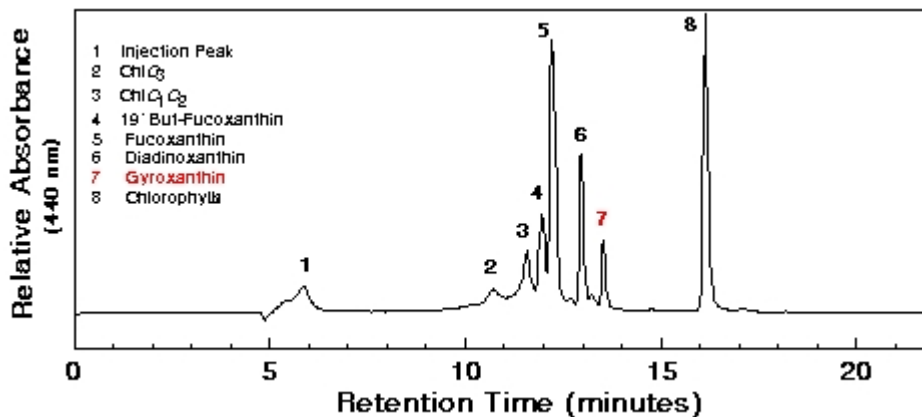


Figure 1. Example chromatogram obtained from a pure culture of *Karenia brevis* using the protocols outlined above.