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Validation of significant wave height retrieval from co-polarization Chinese Gaofen-3 SAR imagery using an improved algorithm

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Abstract

Chinese Gaofen-3 (GF-3) is the first civilian satellite to carry C-band (5.3 GHz) synthetic aperture radar (SAR). During the period of August 2016 to December 2017, 1 523 GF-3 SAR images acquired in quad-polarization (vertical-vertical (VV), horizontal-horizontal (HH), vertical-horizontal (VH), and horizontal-vertical (HV)) mode were recorded, mostly around China’s seas. In our previous study, the root mean square error (RMSE) of significant wave height (SWH) was found to be around 0.58 m when compared with retrieval results from a few GF-3 SAR images in co-polarization (VV and HH) with moored measurements by using an empirical algorithm CSAR_WAVE. We collected a number of sub-scenes from these 1 523 images in the co-polarization channel, which were collocated with wind and SWH data from the European Centre for Medium-Range Weather Forecasts (ECMWF) reanalysis field at a 0.125° grid. Through the collected dataset, an improved empirical wave retrieval algorithm for GF-3 SAR in co-polarization was tuned, herein denoted as CSAR_WAVE2. An additional 92 GF-3 SAR images were implemented in order to validate CSAR_WAVE2 against SWH from altimeter Jason-2, showing an about 0.52 m RMSE of SWH for co-polarization GF-3 SAR. Therefore, we conclude that the proposed empirical algorithm has a good performance for wave retrieval from GF-3 SAR images in co-polarization.

Key words: Gaofen-3, synthetic aperture radar, significant wave height


1 Introduction

It is well known that synthetic aperture radar (SAR) has the capability of wind and wave monitoring (Chapron et al., 2001) in large swath coverage with a fine spatial resolution, especially in extreme sea states (Li et al., 2002; Hwang and Fois, 2015; Li, 2015; Shao et al., 2017a). To date, SAR data is available at C-band (5.3 GHz) Canadian Radarsat-2 (R-2), and European Sentinel-1 (S-1); X-band (9.8 GHz) German TerraSAR-X/TanDEM-X, Italian Cosmo-SkyMed and Korean Kompsat-5; and L-band (1.2 GHz) Japanese ALOS-2 satellite. Gaofen-3 (GF-3) SAR at C-band was launched by the China Academy of Space Technology (CAST) in August 2016, and can operate in 12 imaging modes with a fine spatial resolution of image up to 1 m. It has a 755-km orbit height above the earth’s surface with a 26-day repeat cycle. Recently, preliminary analysis of marine applications using GF-3 SAR data have been achieved, in particular, for wind (Wang et al., 2017; Ren et al., 2017; Shao et al., 2018) and wave monitoring (Yang et al., 2017; Shao et al., 2017b).

Based on a good understanding of the wave imaging mechanism on SAR, including tilt modulation (Lyzenga, 1986), hydrodynamic modulation (Feindt et al., 1986) and velocity bunching (Alpers et al., 1981; Alpers and Bruening, 1986), wave retrieval algorithms have been thoroughly studied over recent decades. Basic scattering physics is widely used in theoretical-based wave retrieval algorithms, e.g., Max-Planck Institute Algorithm (MPI) (Hasselmann and Hasselmann, 1991; Hasselmann et al., 1996), the semi parametric retrieval algorithm (SPRA) (Hasselmann and Hasselmann, 1991; Hasselmann et al., 1996), the semi parametric retrieval algorithm (SPRA) (Mastenbroek and De Valk, 2000), the parameterized first-guess spectrum method (PFSM) (Sun and Guan, 2006; Shao et al., 2015; Lin et al., 2017) and the partition rescaling and shift algorithm (PARSA).
(Schulz-Stellenfleth et al., 2005; Li et al., 2010), which are independent of radar frequency and imaging polarization. However, velocity bunching is a non-linear modulation, that causes waves of a shorter than specific wavelength to be undetectable in the azimuth direction (or satellite flight direction) and a cutoff in the SAR intensity spectrum (Alpers and Brüning, 1986; Hasselmann and Hasselmann, 1991). The idea behind these theoretical-based algorithms is directly inverting the SAR intensity spectrum into the wave spectrum after employing a "first-guess" wave spectrum, which is considered to be the compensation for loss in the SAR intensity spectrum due to non-linear effect of velocity bunching. The algorithms MPI and PARSATake the simulation from a numeric wave model, while a prior wave spectrum is produced by using a parameterized empirical function in the schemes of algorithms SPRAT and PFSM, such as the Jonswap spectrum (Hasselmann and Hasselmann, 1985). Therefore, they are limitedly applied in the operation system, because the quality of the "first-guess" wave spectrum determines the SAR-derived wave spectrum. Moreover the "first-guess" wave spectrum is not reliable in the presence of other marine phenomena. Ocean wave parameters, e.g., significant wave height (SWH) and mean wave period (MWP), are calculated from the SAR-derived wave spectrum.

An empirical wave retrieval algorithm for C-band ERS SAR is proposed by Schulz-Stellenfleth et al. (2007), denoted as CWave_ERS. In particular, CWave has been tuned for ENVISAT Advanced SAR (ASAR) (Li et al., 2011) and S-1 SAR (Stopa and Mouche, 2017). The CWave model is designed to be an empirical function, in which the sea state parameter SWH is connected with a set of variables, including normalized radar cross section (NRCS), variance of the normalized SAR image and several orthonormal functions derived from the two-dimensional SAR spectrum. The advantage is that SWH can be directly retrieved from SAR without calculating the modulation transfer function (MTF) of each SAR mapping modulation. However, CWavess have only been validated for SAR data acquired in wave mode until now. Following the idea of CWave, researchers have recently exploited the empirical algorithms XWave for X-band SAR (Bruck and Lehner, 2015; Pleskachevsky et al., 2016; Shao et al., 2017c).

Recent research has revealed that the azimuthal cutoff wavelength is derived to be proportional to the second moment of a wave spectrum (Hasselmann and Hasselmann, 1991; Marghany et al., 2002). On the other hand, SWH is calculated by integrating a wave spectrum according to traditional wave theory. Therefore, SWH is theoretically related to cutoff wavelength in the azimuth direction. Interestingly, several studies have made an attempt to retrieve SWH through azimuthal cutoff wavelength (Wang et al., 2012; Ren et al., 2015; Grieco et al., 2016; Stopa et al., 2016). The dependences of radar incidence angle and wave propagation direction on azimuthal cutoff wavelength were investigated in our previous study using theoretical analysis and simulation experiment (Shao et al., 2016). Then we constructed an empirical wave retrieval algorithm, denoted as CSAR_WAVE, which was tuned through VV-polarization S-1 SAR image and collocated measurements from the National Data Buoy Center (NDBC) buoys of the National Oceanic and Atmospheric Administration (NOAA). The preliminary assessment showed that CSAR_WAVE is applicable for GF-3 SAR with around 0.58 m root mean square error (RMSE) of the retrieved SWH compared with the NDBC buoy measurements of NOAA (Shao et al., 2017b). However, the accuracy of the retrieval results is expected to be further improved for the operational application of GF-3 SAR, as the SAR-derived product is dedicated to oceanography research, especially in coastal waters. Therefore, in this study, we have developed an improved wave retrieval algorithm for GF-3 SAR in co-polarization (VV and HH).

The remaining part of this paper is organized as follows: collected datasets are briefly described in Section 2. Section 3 shows the methodology of derivation of the empirical algorithm. In this section, the process of tuning the empirical algorithm for co-polarization GF-3 SAR is also presented. Then the validation of the retrieved SWHs using the proposed algorithm and other three existing empirical algorithms, is shown in Section 4. Conclusions are summarized in Section 5.

2 Description of dataset

Since GF-3 SAR was launched in 2016 by CAST, during the period of August 2016 to December 2017 a number of images acquired in quad-polarization mode (QPS-1/II) (vertical-vertical (VV), horizontal-horizontal (HH), vertical-horizontal (VH), and horizontal-vertical (HV)) have been recorded. Most of these GF-3 SAR images were located around China’s seas and they were processed as Level-1A (L-1A) products, which have a standard pixel of 8 m and 25 m for QPS-1 and QPS-II mode, respectively. Because the SAR backscattering signature from a sea surface in copolarization is more sensitive than that in cross-polarization (VH and HV), the collected GF-3 SAR images in VV- and HH-polarization channel are used in our study. Equation 1 is used for calculating the NRCS of a co-polarization GF-3 SAR intensity image.

$$\phi^0 = DN^2 \left( \frac{M}{32767} \right)^2 - N,$$  \hspace{1cm} (1)

where $\phi^0$ is the NRCS united in db, $DN$ is the SAR-measured intensity, $M$ and $N$ are the calibrated constants stored in the annotated file with the original SAR intensity image.

As an example, a quick-look image of the calibrated GF-3 SAR image acquired in QPS-1 mode at 10:40 UTC on 18 January 2017 in VV- and HH-polarization is shown in Fig. 1a and Fig. 1b, respectively. It was found that wind direction is vertical to two-dimensional SAR image spectra for wavelengths between 800 m and 3 000 m at peaks (Alpers and Brümmer, 1994), indicating wind direction can be directly measured from SAR. However, the SAR-derived wind direction has a 180° ambiguity. The European Centre for Medium-Range Weather Forecasts (ECMWF) provides global reanalysis wind data with a fine spatial resolution of 0.125°×0.125° at intervals of six hours, which is employed to remove that ambiguity. The wind speed ($U_10$) at 10 m-height above sea surface can be inverted by using the combination method proposed in our previous study (Shao et al., 2014), which is based on the geophysical model function (GMF) CMOD5 (Hersbach et al., 2007) and CMOD4 (Stoffelen and Anderson, 1997). The colored vectors shown in Fig. 1 represent the SAR-derived wind fields. Note that it is necessary to use the polarization ratio (PR) at C-band (Zhang et al., 2011) together with GMF to retrieve the wind field from an HH-polarization GF-3 SAR image.

In our study, all the GF-3 SAR images are divided into a number of sub-scenes with a spatial coverage of about 5 km×5 km. These extracted sub-scenes are collocated with 0.125° gridded ECMWF reanalysis wave data at intervals of six hours. It is necessary to ensure that the sub-scenes covering the locations of the ECMWF reanalysis grids data are calculated by bilinear interpolation in temporal scale, as there is a time difference between the GF-3 SAR imaging time and the interval time of the ECMWF
reanalysis grids data. Then we have more than ten thousand matchups, which are treated as a dataset for tuning an improved algorithm for wave retrieval from GF-3 SAR images. Figure 2 shows the ECMWF reanalysis wind and wave map at 06:00 UTC on 18 January 2017, in which the black rectangle represents the spatial coverage of a GF-3 SAR image located in the South China Sea as exhibited in Fig.1. It should be noted that the SWH from the ECMWF reanalysis data goes up to 4 m, therefore, GF-3 SAR images at low to moderate sea states are included in the dataset.

Recently, a new approach for SWH retrieval in hurricanes has been constructed through studying the relationship between SWH and NRCS (Romeiser et al., 2015). As mentioned by the authors, this is still to be improved due to the complicated non-linear effect of waves at extreme sea states.

The high-precision ocean altimetry on Jason-2 launched in 2008 is a marine observation system over global sea, which is a follow-on satellite of the oceanography monitoring mission of Jason-1. So far, Operational Geophysical Data Record (OGDR) derived from the Jason-2 satellite track is a near real-time operational product, in particular including more reliable SWH data which is better than that of Jason-1 by about 7% (Abdalla et al., 2010). This high-quality product is essentially dedicated to oceanography research. An additional 91 quad-polarization GF-3 SAR images were collected and these GF-3 SAR images cover the footprints of altimeter Jason-2, which were implemented in order to validate the improved algorithm in our study.

3 Methodology

In this section, the methodology of derivation of an improved algorithm is presented, which is based on two existing empirical wave retrieval algorithms CWAVE and CSAR_WAVE. Then the improved algorithm, denoted CSAR_WAVE2, is tuned for co-po-
Algorithm CWAVE

As mentioned in Section 1, algorithms MPI, SPRA, PFSM and PARSA rely on prior information on a wave spectrum, e.g., numeric simulation from a wave model and computation from a parametric wave function. In the operational application, they take some time to produce a “first-guess” wave spectrum and on the non-linear inversion of an SAR spectrum into a wave spectrum (Hasselmann and Hasselmann, 1991; Hasselmann et al., 1996). Moreover, it is difficult to improve the accuracy of SWH retrieval in the physics aspect of theoretical-based algorithms. In practice, empirical models are routine operations for marine applications of Scatterometer and SAR, such as GMF’s for wind retrieval (Stoffelen and Anderson, 1997; Hersbach et al., 2007). The GMF CWAVE family, e.g., CWAVE, ERS (Schulz-Stellenfleth et al., 2007) for ERS SAR and CWAVE_ENV (Li et al., 2011) for ENVISAT-ASAR, were originally exploited by the SAR oceanography group at the German Aerospace Center (DLR), which allows for direct retrieval of wave parameters from SAR wave mode data without calculating the complex MTF of each SAR mapping modulation.

In a SAR image, sea state measurement $S$ can be determined by a set of imaging parameters $s_i$ ($s_1, s_2, ..., s_n$) with a coefficient vector $a_i$ ($a_{i0}, a_{i1}, ..., a_{in}$). Due to the modulation of velocity bunching, non-linearity among different imaging parameters is also included by adding the products of different imaging parameters $s_i$ with a coefficient vector $a_{ij}$ ($i \leq j \leq n$). Based on this assumption, the function of CWAVE principally follows the multiple-regression method stated as:

$$S = a_0 + \sum_{i=1}^{n} a_i s_i + \sum_{i,j=1}^{n} a_{ij} s_i s_j.$$  

In the CWAVE models, imaging parameters $s_i$ include NRCS $\sigma_0$ and variance of the normalized SAR image $cvar$, both of which directly contribute to sea state, and a set of orthonormal functions derived from the two-dimensional SAR spectrum. $cvar$ is defined as follows:

$$cvar = \text{var}\left(\frac{I - \overline{I}}{I}\right),$$

where $I$ is the pixel intensity of a SAR image and $\overline{I}$ is the average of $I$. The coefficients in CWAVE models were tuned for ERS and ENVISAT-ASAR wave mode data acquired in VV-polarization at a fixed incidence angle of 23°. Therefore, CWAVE needs to be re-tuned for other SAR data at various incidence angles, such as CWAVE_SI for S-1 SAR (Stopa and Mouche, 2017).

Algorithm CSAR_WAVE

The relationship between cutoff wavelength in azimuth direction $\lambda_c$ and SWH was demonstrated in the study proposed by Hasselmann and Hasselmann (1991):

$$\lambda_c = \frac{\pi \beta}{\sqrt{\int \left| T_\omega \right|^2 S_\omega d\omega}},$$

where $\beta$ is the satellite range-to-velocity parameter, $|T_\omega|^2$ is the velocity bunching transfer function, $\omega$ is wave frequency and $S_\omega$ is the one-dimensional wave spectrum. In the imaging process, $\lambda_c$ can be estimated by fitting a one-dimensional SAR spectrum with a Gaussian fit function (Sun and Kawamura, 2009). The Gaussian fit function has the formulation $\exp\{\pi (k/k_c)\}$, in which $k_c$ is the azimuthal wavenumber and $k_c = 2\pi/\lambda_c$ is the azimuthal cutoff wavenumber. Through analyzing a number of recorded ENVISAT-ASAR wave mode data, recent research has revealed that $\lambda_c$ provides meaningful information about the sea state, even at large sea states (>250 m) (Stopa et al., 2016).

SWH can be calculated by integrating wave spectrum $S_\omega$:

$$SWH = \frac{4}{\pi^2} \int S_\omega d\omega.$$  

Theoretically, SWH is related to $\lambda_c$ through the above two equations. Recently, several algorithms have been developed by using the $\lambda_c$ to estimate SWH for ENVISAT-ASAR (Wang et al., 2012), quad-polarization R-2 SAR (Ren et al., 2015) and S-1 SAR (Grieco et al., 2016; Stopa and Mouche, 2017).

The dependency of $\lambda_c$, radar incidence angle $\theta$ and peak wave direction relative to range direction $\varphi$ on SWH was simulated through the widely used Jonsstedt wave spectrum model (Hasselmann and Hasselmann, 1985). It was found that SWH is linearly related with $\lambda_c/\beta$, while SWH has a positive and negative relationship with $\theta$ and $\varphi$ respectively (Shao et al., 2016). The semi-empirical wave retrieval algorithm, denoted as CSAR_WAVE, was originally developed for S-1 SAR in our previous study. The formulation of CSAR_WAVE is designed as a first-order linear function,

$$SWH = \left(\frac{\lambda_c}{\beta}\right) (A_1 + A_2 \sin \theta + A_3 \cos 2\varphi) + A_4,$$

where coefficients $A$ are determined from S-1 SAR image collocated NDBC buoys of NOAA. It was reported by Shao et al. (2017b) that the RMS of SWH is 0.58 m and 0.57 m when using CSAR_WAVE for GF-3 SAR in VV- and HH-polarization respectively, as the retrieved SWHs are validated against the NDBC buoys of NOAA around U.S. waters.

Tuning the improved algorithm

In order to enhance the sensitivity of non-linearity on SWH in an empirical algorithm, the formulation of a CWAVE model is basically employed. However, imaging parameters $s_i$ in the CWAVE model are set as a vector $(U_{10}, \alpha_0, cvar, \lambda_c/\beta, \sin \theta, \cos 2\varphi, \lambda_{\text{SAR}})$ for practical application, in which three factors, e.g., $U_{10}$, $\sigma_0$ (unitless in dB) and $cvar$, are directly related with sea state (Li et al., 2010; Grieco et al., 2016; Stopa et al., 2016). Besides, the dependences of other factors on SWH, including $\lambda_c/\beta$, $\theta$ and $\varphi$, have been already investigated by Shao et al. (2016). In particular, $\lambda_{\text{SAR}}$ represents the SAR length at peaks of the SAR spectrum, which is also assumed to be an essential factor in SWH, according to the derivation model through the SAR imaging mechanism of ocean wave, as referred to in Eq. (16) proposed by Wang et al. (2012).

In total, we have obtained more than ten thousand sub-scenes extracted from GF-3 images in co-polarization channel with collocated ECMWF reanalysis SWH data. During the process, three variables, i.e., $\lambda_c$, $\varphi$ and $\lambda_{\text{SAR}}$, were derived from the SAR intensity spectrum. The sub-scene extracted from the case exhibited in Fig. 1, which is acquired in VV-polarization, is shown in Fig. 3a. The corresponding two-dimensional SAR spectrum of the sub-scene is shown in Fig. 3b, in which $\varphi$ and $\lambda_{\text{SAR}}$ can be dir-
The Gaussian fitted result of $\lambda_c$ is illustrated in Fig. 3c. The matchup dataset is used to determine the 36 coefficients $a_{ij}$ ($i \leq j \leq 7$) in Eq. (2) by using the least-squares method, in which subscripts (1, 2, ..., 7) represent the corresponding variables ($U_{10}$, $\sigma_0$, $c_{var}$, $\lambda_c$/$\beta$, $\sin \theta$, $\cos^2 \lambda_{SAR}$), e.g., $a_{12}$ is the coefficient for the term of $U_{10} \times \sigma_0$. The tuned results in the improved algorithm, denoted as CSAR_WAVE2, are shown in Table 1 for co-polarization GF-3 SAR.

Figure 4 shows the fitting results of CSAR_WAVE2 compared with ECMWF reanalysis SWH in our data collection. It is found that the correlation (COR) between the ECMWF reanalysis data and the simulated values is around 0.72 for co-polarization GF-3 SAR. Under these circumstances, we think the improved algorithm CSAR_WAVE2 is suitable for SWH retrieval from co-polarization GF-3 SAR images.

**4 Validation**

With reference to the application process of existing CWAVE and CSAR_WAVE models, the process of SWH retrieval by our use of CSAR_WAVE2 is roughly illustrated in Fig. 5. We first show the quick-look image of the VV-polarization GF-3 SAR image acquired at 20:49 UTC on 26 July 2017 in Fig. 6a. The inverted wave map for this case when using CSAR_WAVE2 is shown in Fig. 6b, in which the several small colored rectangles represent the SWH data measured from the altimeter Jason-2 footprints. It is found that the SAR-derived SWH is close to the SWH data of Jason-2. In particular, the trend of the inverted wave map is consistent with that following the track of the Jason-2 footprints.

In addition, we have applied CSAR_WAVE2 to a total of 91 available GF-3 SAR images and compared the results with those from the SWH data from altimeter Jason-2. In Fig. 7, the RMSE of SWH is 0.51 m for VV-polarization and the RMSE of SWH is 0.52 m for HH-polarization. The reported accuracy of SWH for C-band SAR is an RMSE of SWH of 0.55 m as validated against measurements from moored buoys using the PFSM algorithm (Lin et al., 2017) and RMSE is 0.51 m when comparing the wave retrievals with the WAM model predictions (Schulz-Stellenfleth et al., 2005) using the PARSA algorithm. It is indicated that CSAR_WAVE2 has a better accuracy of SWH retrieval than that using theoretical-based algorithms. In particular, it is applicable without calculating the complex MTF of each mapping modulation.

We also compared the SAR-derived results with SWH from Jason-2 by using the existing three empirical algorithms proposed by Wang et al. (2012), Ren et al. (2015) and Grieco et al. (2016). All of these algorithms were developed based on azimuthal cutoff wavelength and tuned through R-2 and S-1 SAR data acquired in VV-polarization. Figure 8 shows that the RMSE of SWH is 0.70 m, 0.62 m and 0.61 m using the algorithms by Wang et al. (2012), Ren et al. (2015) and Grieco et al. (2016), respectively. And a comparison between SAR-derived SWHs and measure-
ments from NDBC buoys of NOAA shows an approximate 0.58 m RMSE of SWH for co-polarization using CSAR_WAVE (Shao et al., 2017b). This analysis shows that these algorithms all perform less well than the results achieved using CSAR_WAVE2, when non-linear higher-order corrections on sea state are included in CSAR_WAVE2. Therefore, it is recommended that CSAR_WAVE2 is applied operationally for wave retrieval from GF-3 SAR images in co-polarization. However, it is necessary to establish that there are no available data at high sea states in the fitting and validation procedure. CSAR_WAVE2 is expected to be further adopted for high sea states as the non-linearity is higher than at low and moderate sea states, especially in typhoons and hurricanes.

<table>
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<th>VV-polarization, HH-polarization</th>
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Note: The subscripts (1, 2, ..., 7) represent the corresponding variables ($U_{10}, \sigma_0, \text{cvar}, \lambda_c/\beta, \sin\theta, \cos^2\phi, \lambda_{SAR}$), e.g., $a_{12}$ is the coefficient for the term of $U_{10}\sigma_0$.

Fig. 4. Simulated results by using the empirical algorithm CSAR_WAVE2 vs. SWH from ECMWF reanalysis data for 0.1 m of SWH bins between 0 m and 3 m. a. VV-polarization and b. HH-polarization.
In the preliminary assessment (Shao et al., 2017b), the RMSE of SWH was around 0.58 m for GF-3 SAR when using the empirical wave retrieval algorithm CSAR_WAVE as validated against buoy measurements, which was tuned for S-1 SAR in VV-polarization. As for the operational application of GF-3 SAR, it is essential to reduce the retrieval error of the SWH for oceanic and coastal monitoring.

In this study, 1 523 GF-3 SAR images acquired in quad-polarization mode were collected during the period of August 2016 to December 2017. More than ten thousand sub-scenes from these images in the co-polarization channel were collocated with SWH from ECMWF reanalysis data at a 0.125° grid with SWH up to 4 m. Through the dataset, an improved wave retrieval algorithm for GF-3 SAR, denoted as CSAR_WAVE2, was developed. Seven variables, which are explicitly related to sea state and can be directly obtained from a SAR image, were selected for the CSAR_WAVE2 model. CSAR_WAVE2 is more than an updated version of CSAR_WAVE, as the formulation of function has been rigorously redesigned and non-linear higher-order corrections on sea state have been implemented. The COR is 0.72 and 0.71 for VV- and HH-polarization respectively, when the simulated SWH using CSAR_WAVE2 is compared with ECMWF reanalysis SWH data, indicating that CSAR_WAVE2 can be applied for wave retrieval from GF-3 SAR image in co-polarization.

An additional 92 GF-3 SAR images were collected, which cover the footprint of the altimeter Jason-2 mission. Validation shows that the RMSE of SWH is 0.51 m and 0.52 m for GF-3 SAR in VV- and HH-polarization respectively. We also compared the retrieval results with SWH of Jason-2 using three existing empirical algorithms (Wang et al., 2012; Ren et al., 2015; Grieco et al., 2016), showing a 0.60–0.70 m RMSE of SWH. As a result, it is concluded that the accuracy of retrieved SWH from co-polarization GF-3 SAR has been significantly improved using CSAR_WAVE2 at low to moderate sea states.

The applicability of CSAR_WAVE2 will be further investigated.

Fig. 5. The concise flowchart of SWH retrieval using CSAR_WAVE2, with reference to the application process of existing CWAVE and CSAR_WAVE models.

Fig. 6. The quick-look image of the VV-polarization GF-3 SAR image acquired at 20:49 UTC on 26 July 2017 (a) and the inverted wave map of this case using CSAR_WAVE2, in which the several small colored rectangles represent the SWH data measured from altimeter Jason-2 footprints (b).

5 Summary and conclusion

In the preliminary assessment (Shao et al., 2017b), the RMSE of SWH was around 0.58 m for GF-3 SAR when using the empirical wave retrieval algorithm CSAR_WAVE, as validated against buoy measurements, which was tuned for S-1 SAR in VV-polarization. As for the operational application of GF-3 SAR, it is essential to reduce the retrieval error of the SWH for oceanic and coastal monitoring.

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The applicability of CSAR_WAVE2 will be further investigated.
for various GF-3 SAR data, e.g., Spotlight Mode (SL), Standard Stripmap (SS), Wide Scan (WSC), Global Observing Mode (GLO) and Wave Mode (WAV). Recently, GF-3 SAR has captured several typhoons by the National Ocean Satellite Application Center (NSOAS) around China’s seas. Therefore, the applicability of CSAR_WAVE2 will be further investigated and can be adopted for high sea states in the near future.

Fig. 7. SWH from Jason-2 vs. SAR-derived SWH from 92 GF-3 SAR images using CSAR_WAVE2. a. VV-polarization and b. HH-polarization.

Fig. 8. SWH from Jason-2 vs. SAR-derived SWH from 92 VV-polarization GF-3 SAR images using three empirical algorithms, e.g., the results using the algorithm proposed by Wang et al. (2012) (a), the results using the algorithm proposed by Ren et al. (2015) (b), the results using the algorithm proposed by Grieco et al. (2016) (c).
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References

Comparison of two Bayesian-point-estimation methods in multiple-source localization

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Abstract

Environmental uncertainty represents the limiting factor in matched-field localization. Within a Bayesian framework, both the environmental parameters, and the source parameters are considered to be unknown variables. However, including environmental parameters in multiple-source localization greatly increases the complexity and computational demands of the inverse problem. In the paper, the closed-form maximum-likelihood expressions for source strengths and noise variance at each frequency allow these parameters to be sampled implicitly, substantially reducing the dimensionality and difficulty of the inversion. This paper compares two Bayesian-point-estimation methods: the maximum a posteriori (MAP) approach and the marginal posterior probability density (PPD) approach to source localization. The MAP approach determines the sources locations by maximizing the PPD over all source and environmental parameters. The marginal PPD approach integrates the PPD over the unknowns to obtain a sequence of marginal probability distribution over source range or depth. Monte Carlo analysis of the two approaches for a test case involving both geoaoustic and water-column uncertainties indicates that: (1) For sensitive parameters such as source range, water depth and water sound speed, the MAP solution is better than the marginal PPD solution. (2) For the less sensitive parameters, such as, bottom sound speed, bottom density, bottom attenuation and water sound speed, when the SNR is low, the marginal PPD solution can better smooth the noise, which leads to better performance than the MAP solution. Since the source range and depth are sensitive parameters, the research shows that the MAP approach provides a slightly more reliable method to locate multiple sources in an unknown environment.

Key words: source localization, Bayesian-point-estimation method, uncertain environment


1 Introduction

Matched-field processing (MFP), an approach for solving inverse problems by matching acoustic fields measured at an array of sensors with solutions of the wave equation, has been developed for localizing acoustic sources (Bucker, 1976) and for estimating acoustic parameters (Tolstoy and Diachok, 1991). For source localization problems, MFP estimates source ranges and depths by comparing acoustic fields with replica fields computed for a grid of possible source locations using an acoustic field model. Localization requires good knowledge of the physical properties of the ocean environment and the tilt of the array, which strongly affect the propagation of acoustic signals. Two challenging issues in MFP involve source localization when properties of the environment and the array are poorly known, and localization of multiple sources. This paper addresses both of these problems.

Variants of MFP have been proposed for localization of multiple sources. Model-based methods in underwater environments were discussed (Greening et al., 1997; Nielsen, 2005). These multiple source localization techniques typically rely on eigenvector decompositions, modified Bartlett functions, or combination of these two methods. In a recent work, Michalopoulou (2006) developed a simultaneous approach to multiple-source localization based on formulating the PPD over source locations, noise variance, and complex source strengths, and Gibbs sampling these parameters to provide optimal estimates and uncertainties of the results. Nevertheless, it was also shown that the approach is highly sensitive to environmental uncertainties. Dosso and Wilmurt (2011) developed two approaches to source localization called marginalization and focalization. Marginalization includes first integrating the PPD over the environmental unknown parameters to obtain a sequence of joint marginal probability distributions over source range and depth. Focalization includes determining the source location that maximizes the PPD over all source and environmental parameters, that is, the MAP solution. Dosso indicates that marginalization significantly outperforms focalization for source localization in an unknown environment. However, through these paper’s analysis, the MAP method provides a slightly more reliable result than the marginal PPD approach, which are some different from Dosso’s conclu-
sions. A major reason is that, focalization and marginalization use different numerical algorithms to this optimization problem, which is the main reason that makes the marginalization approach outperforms the focalization approach significantly. Specifically, the marginalization approach has applied a powerful Markov-chain Monte Carlo method, here which combines Metropolis–Hastings Gibbs sampling (GS) for environmental parameters and heat-bath GS for source ranges and depths. However, the focalization approach has used both differential evolution and adaptive simplex simulated annealing as the numerical optimization. So the difference between focalization and marginalization is that they use different numerical algorithms rather than two different Bayesian-point-estimation methods, that is, the MAP solution and the marginalization PPD solution. Although the MAP approach and the marginalization PPD approach generally provide different solutions to source localization in an uncertain environment, there does not appear to have been any comparison of the two approaches to date, this paper compares the two approaches for the multiple-source localization problem, and shows the physical reason of the MAP approach being better than the marginalization PPD approach.

In this paper, the genetic algorithms (GA) are used for the optimization. To overcome the precarious problem of the GA, a temperature is adopted, as in simulated annealing, giving the opportunity to stretch the probability and enhance the algorithm performance. But, as with simulated annealing, the choice of the temperature \( T^* \) is difficult. It must be neither too high nor too low. A good compromise is a temperature of the same magnitude as the objective function \( \Phi(m) \), here \( T^* = \min \| \Phi(m) \| \). During optimization, the objective function increases and the temperature reduce. Meanwhile, the closed-form maximum-likelihood expressions for source strengths and noise variance at each frequency allow these parameters to be sampled implicitly, substantially reducing the dimensionality and complexity of the inversion.

This paper compares the MAP approach and the marginal PPD approach to multiple-source localization problem. The MAP approach determines the sources locations by maximizing the PPD over all source and environmental parameters just like focalization. The marginal PPD approach integrates the PPD over the unknowns to obtain a sequence of one-dimension (1D) marginal probability distribution over source range or depth something like marginalization. The MAP method and the marginal PPD approach represent distinct approaches in estimating parameters of interest in the presence of nuisance parameters and generally produce different solutions for nonlinear problems such as acoustic inversion. When considering a nonlinear problem, the solutions via these two methods are not coincident.

The two approaches are illustrated conceptually in Fig. 1, which considers determining the value of a model parameter of interest, \( m_1 \) and \( m_2 \). Figure 1a considers the case of a binary Gaussian distribution. It is essentially a probability density function of a binary gaussian distribution:

\[
\sigma(m_1, m_2) = 3(1 - m_1)^2 e^{-m_1^2 - (m_2 + 1)^2} - 10 \times \\
\left( \frac{1}{5} m_1 - m_1^3 - m_2^3 \right) e^{-m_1^2 - m_2^2} - \frac{1}{3} e^{-(m_1 + 1)^2 - m_2^2}.
\]

Figure 1b shows the MAP solution for parameter \( m_1 \) and \( m_2 \), which are \(-0.19\) and \(1.63\). Figure 1c shows the corresponding marginal distribution for \( m_1 \) obtained by integration over \( m_2 \), whose solution is \(-0.38\). The marginal PPD solution for parameter \( m_1 \) in Fig. 1d is \(1.58\). Obviously, the MAP solution and the marginal PPD approach end up with different results. Although the MAP approach and the marginal PPD approach generally provide different solutions to source localization in an uncertain environment, it is impossible to prove in theory which of these
two methods is more reliable. Monte Carlo studies are employed in both localization approaches which are applied to a large number of noisy synthetic data. Statistical analysis of the results is carried out, and indicates that the MAP approach provides a slightly more reliable approach to locate multiple sources in an unknown environment.

In the paper, the number of sources was assumed known and set equal to two. In practice, there is no such precise information on the number of sources. There are several ways of determining the source numbers, and we studied three source number estimation algorithm based on information theoretic criterion and another method based on Gershgorin Disks theory, and compares their performance based on numerical simulation results. It is found that the algorithms based on the Bayesian information criterion (BIC) are suitable for the white Gaussian noise. However, due to the limited space of the article, this paper only discusses the multiple-source localization after knowing the number of sources.

2 Theory and algorithms

In a Bayesian formulation (Dosso and Wilmut, 2011), the solution to an inverse problem is characterized by its PPD. Before experiment, the information about the models is reflected in the a priori distribution ρ(m), but after the experiment, the information about the models is reflected in the a posteriori distribution ρ(m|d). These two distributions are related through the likelihood function L(m|d), which is a measure of the fitness between the measured data and the data generated using an acoustic field model and the unknown parameters m (Bayesian Theorem):

\[ L(m|d) = \frac{1}{\sqrt{2\pi\sigma(m)}} \exp\left(-\frac{1}{2\sigma(m)} \right) \]  

where the likelihood function is \( L(m|d) \propto \exp[-E(m)] \), and \( E(m) \) is the data misfit function. The objective function \( \varphi(m) \) can be written as

\[ \varphi(m) = E(m) - \log \rho(m). \]  

Due to multi-dimensionality, the a posteriori distribution is not fit for graphic display, and therefore mainly integral properties of the a posteriori distribution are of interest, such as the MAP solution, and the marginal PPD for parameters \( m \), defined respectively as

\[ m_{\text{MAP}} = \arg \left( \max \left( \varphi(m) \right) \right), \quad m \in M, \]  

\[ \sigma^2(m) = \int \sigma(m) dm_1 \cdots dm_{i-1} dm_{i+1} \cdots dm_M, \]  

where \( M \) is the number of the estimated parameters. The MAP solution is found using the optimization algorithm without integration, which consumes less time. The main advantage of the marginal probability distribution is that it provides a quantitative measurement of localization uncertainty.

To define the data misfit function \( E(m) \), consider data \( \mathbf{p}^f = \{ p^f ; f = 1, F \} \) consisting of complex acoustic measurements at \( F \) frequencies and \( N \) hydrophones. Namely, \( \mathbf{p}^f = \{ p^f_{n,s} ; n = 1, N \} \) is a complex vector with \( N \) elements. The acoustic measurements at each frequency is assumed to be due to \( N \) sources at locations \( x = \{ x_s = (r_s, z_s) ; s = 1, N \} \) with complex source strengths \( S = [S^1] \). The data errors are considered complex Gaussian-distributed random variables with unknown standard deviations \( \mathbf{w} \). The likelihood function is given by

\[ L(m, S, \mathbf{w}) = \prod_{f=1}^{F} \left( 2\pi\sigma^2(m) \right)^{-N} \exp \left[ -\frac{1}{2\sigma^2(m)} \sum_{s=1}^{N} (w(x_s, \omega)| S(x_s, \omega)) \right] \]  

\[ = \frac{1}{\prod_{f=1}^{F} (2\pi\sigma^2(m))^N} \exp \left( \frac{1}{2} \sum_{f=1}^{F} \left[ p^f - D_f S_f \right]^2 / \sigma^2(m) \right) \]  

\[ = \exp \left\{ -\sum_{f=1}^{F} [N\ln(2\pi\sigma^2(m)) + \mid p^f - D_f S_f \mid^2 / \sigma^2(m) \right\}, \]  

where \( \mathbf{w}(x_s, \omega) \) represents the replica acoustic fields computed for a zero-phase unit-amplitude source at \( x_s \) and \( \mathbf{D}_f \) is an \( N \times N \) complex matrix defined as \( \mathbf{D}_f = \mathbf{w}(x_s, \omega) \) and \( \mathbf{S}_f \) is an \( N \times 1 \) complex matrix defined as \( \mathbf{S}_f = S(x_s, \omega) \).

An unknown source can be treated by maximizing the likelihood over \( \mathbf{S} \) and \( \mathbf{\varphi} \) \( \frac{\partial L}{\partial S_f} = \frac{\partial L}{\partial \mathbf{\varphi}} = 0 \) to give

\[ \varphi(m) = \sum_{f=1}^{F} [2N\ln(1 - D_f D_f^H)]^2 + (N + N \ln \sigma - N \ln N], \]  

neglecting additive constants leads to

\[ \varphi(m) \propto \sum_{f=1}^{F} [2N\ln(1 - D_f D_f^H)]^2. \]  

Hence, by using this equation, the corresponding variability in standard deviations and source strengths is accounted for implicitly. This implicit sampling replaces explicit sampling over these two parameters, substantially reducing the dimensionality of the inversion. For an environmental model with \( N \) parameters, explicit sampling of all parameters has the dimension \( 2NF + 2N + N \) whereas implicit sampling reduces this to \( 2NF + N \). Such as, in the latter example which involves two sources at three frequencies and seven environmental parameters, the dimensionality is reduced from 26 to 11.

Knowing that the likelihood function is usually related to the objective function \( \varphi(m) \) through an exponential \( L = \exp(-\varphi(m)/\theta) \) (Gerstoft and Mechenbrauker, 1990), where \( \theta \) is the estimated noise power, the following scaling is used

\[ L_{\text{opt}} = \exp(-\varphi(m) - \varphi(m))/T, \]  

where \( \varphi(m) \) is the objective function mentioned in Eq. (7). \( m \) is the estimated parameter corresponding to the optimal value of the objective function and \( T \) is the temperature (Li, 2016). Researches show that a good value for \( T \) is the average of the 50 best objective functions obtained during the optimization, minus the best value of the objective function. It should be pointed out that this value of \( T \) is not intended to estimate the noise, but rather to provide a reasonable value with which to estimate the uncertainties of the parameters. The advantage is that it works irrespective of the stochastic model for the data.
3 Simulation example

This section illustrates multiple-source localization with a simulated example in a poorly-known environment. The scenario is illustrated in Fig. 2 and parameter values and prior bounds are summarized in Table 1. The locations of the two sources are \((r_1, z_1) = (7\, \text{km}, 60\, \text{m})\) and \((r_2, z_2) = (7\, \text{km}, 20\, \text{m})\), with corresponding SNRs at the receiver array between -10 dB and 15 dB at each of three frequencies of 200 Hz, 300 Hz, and 400 Hz. Simulated acoustic data were recorded by a vertical line array (VLA) which had 24 elements at 3.5-m spacing, sampling the water column with depths of 4–90 m using the normal-mode propagation model SNAP. The prior information for all source locations is a uniform distribution over 10–90 m in depth and 1–10 km range, and the depth and range steps are 0.5 m and 35 m, respectively. Water column unknowns include the water depth \((D)\), and the sound-speed profile represented by three parameters \((c_1\ldots c_3)\) at depths of 0 m, 9.5 m, and \(D\) m. Unknown geoaoustic parameters include the bottom sound speed \((c_b)\), density \((\rho_b)\), and attenuation \((\alpha_b)\). Prior information for the estimated parameters is given in Table 1.

![Fig. 2. Experimental configuration and shallow water environment.](image)

4 The multiple-source localization results

Figure 3 shows inversion results for all parameters to one realization at an SNR of 10 dB. Further, the results are summarized in Table 1. Figure 3 shows that the MAP solution and the marginal PPD solution for range \(r\) and depths \(z\) are the same and slightly smaller than the correct value. While the MAP solution for depths \(z\) are slightly better than the marginal PPD solution. Figure 3 also shows that the MAP solution and the marginal PPD solution for other environmental parameters are nearly the same and slightly differ from the correct value. In conclusion, Fig. 3 shows that the MAP method and the marginal PPD approach are comparable in source localization and environment inversion. Figure 3 also shows that the MAP or marginal PPD methods have the advantage that these methods have narrower main lobe and lower side lobes than conventional MFP (Li, 2016). This is helpful to solve the problem of localizing a weak target with the presence of stronger interference in an uncertainty ocean environment. The weak source of interest cannot be masked by the loud interfering sources presented in coastal waters.

To evaluate and compare the MAP method and the marginal PPD approach to multiple-source localization in an uncertain environment, a Monte Carlo performance study was carried out, with results summarized in Fig. 4. The performance study considers the two sources at a variety of SNRs between -10 and 15 dB. For each case these two approaches were applied to 50 realizations of noisy acoustic data to allow statistical analysis. Localization performance is quantified in terms of the probability of correct (PCL) localization, defined here as achieving absolute errors in source range and depth of less than 400 m and 6 m, respectively, for both sources. Results are given in Fig. 4. Figure 4 shows that the MAP method slightly produces higher PCL values than the marginal PPD approach for SNRs from -10 dB to 10 dB for the first source and most of SNRs for the second source. At the SNR of 15 dB for the first source, and 5 dB and 15 dB for the second source, similar PCL results are obtained for the two methods.

To further evaluate and compare the MAP method and the marginal PPD approach for estimating ocean acoustic parameters, Fig. 5 gives the 95% confidence intervals (CIs) for the inversion results of the two methods. Figure 5 shows that, as the SNR increases, the CIs become narrow and approach the real value gradually. This indicates that the proportion of the correct inversion rises. Figure 5 also shows that in addition to bottom sound speed, the MAP method has equal or a slight advantage over the marginal PPD approach. The same conclusions are also gained in Fig. 3.

In Dosso’s article (Dosso and Wilmot, 2011), the focalization method adopts the MAP solution, and marginalization takes the marginal PPD solution. Dosso indicates that marginalization significantly outperforms focalization for source localization. However, through the above analysis, the MAP method generally produces higher PCL values than the marginal PPD approach, which are some different from Dosso’s conclusions. A major reason is that, focalization and marginalization use different numerical algorithms to this optimization problem, which is the main reason that makes the marginalization approach outperforms the focalization approach significantly. So the difference

| Table 1. Parameter value, prior bounds and inversion results |
|------------------|------------------|------------------|------------------|------------------|
| Parameter        | True values      | Bounds           | The MAP solution | The marginal PPD solution |
| \(r_1/\text{km}\) | 7                | [1, 10]          | 6.8              | 6.8               |
| \(r_2/\text{km}\) | 7                | [1, 10]          | 6.8              | 6.8               |
| \(z_1/\text{m}\)  | 60               | [10, 90]         | 59.6             | 59.6              |
| \(z_2/\text{m}\)  | 20               | [10, 90]         | 19.7             | 19.4              |
| \(D/\text{m}\)    | 100              | [98, 102]        | 98.7             | 98.5              |
| \(t/\text{m}\)    | 0                | [-10, 10]        | -0.16            | -0.16             |
| \(c_0/\text{m/s}^{-1}\) | 1580 | [1520, 1700] | 1592.0 | 1592.7 |
| \(\rho_0/\text{g/cm}^3\) | 1.5  | [1.2, 2.2] | 1.5  | 1.6    |
| \(\alpha_0/\text{dB/λ}^{-1}\) | 0.1 | [0.0, 0.5] | 0.19 | 0.23 |
| \(c_1/\text{m/s}^{-1}\) | 1520 | [1515, 1525] | 1519.6 | 1518.5 |
| \(c_2/\text{m/s}^{-1}\) | 1518.8 | [1514, 1522] | 1518.4 | 1519.3 |
| \(c_3/\text{m/s}^{-1}\) | 1510 | [1508, 1512] | 1509.2 | 1509.9 |
between focalization and marginalization is that they use different numerical algorithms rather than two different Bayesian-point-estimation methods, that is, the MAP solution and the marginal PPD solution.

In order to eliminate the influence of different numerical algorithms on localization, both of the two methods mentioned in this paper have used the genetic algorithm as optimization algorithm. To further compare the MAP solution and the marginal PPD solution, Fig. 6 gives the root-mean-square error (Rmse) for both methods. The Rmse is defined as

$$\sigma = \sqrt{\frac{\sum_{i=1}^{n} (M_i^e - M_i^t)^2}{n}},$$  \hspace{1cm} (9)$$

where $n$ is the number of realization ($n=50$), $M_i^e$ is the estimated value for unknown parameters, and $M_i^t$ is the true value. The Rmse can well measure the deviation between the estimate and the truth value, and reflect the precision of the method. Figure 6 gives the root-mean-square error of the MAP solution and the marginal PPD solution. The results can be divided into three

![Fig. 3. The marginal PPDs for all parameters. The red line indicates the MAP solution, and the blue line the true value.](image)

![Fig. 4. Localization performance study for two sources. Probability of correct localization over 50 noise realizations is shown for the MAP method (open circles) and the marginal PPD approach (dotted line) as a function of the SNR. The first image indicates PCL results for the first source, and the second image indicates PCL results for the second source.](image)
5 Discussion and conclusions

This paper considered the MAP method and the marginal PPD approach to multiple-source localization when uncertain environmental parameters are included as unknowns in an augmented inverse problem. The MAP method and the marginal PPD approach represent distinct approaches in estimating parameters of interest in the presence of nuisance parameters and generally produce different solutions for nonlinear inverse problems. Hence, Monte Carlo analysis was applied to compare the two approaches for source localization in an uncertain environment. Both approaches were applied to a large number of noisy synthetic data sets for a test case involving uncertain water-column and seabed parameters.

In the MAP method, the PPD is maximized numerically over all dimensions to provide the most probable set of model parameters, including optimal source ranges and depths. The MAP solution is found using the optimization algorithm without integration, which takes less time but has no measure of uncertainty. In the marginal PPD approach, the PPD is integrated numerically to produce 1D marginal probability distributions over source range or depth, from which source locations can be obtained. In addition, it provides uncertainty analysis, which aids in understanding the information content of the inverse problem. But this method increases computational effort (the marginal PPD approach generally takes about five times as long as the MAP method).

Statistical analysis indicates that: (1) for sensitive parameters...
such as source range, water depth and water sound speed, the MAP solution is better than the marginal PPD solution; (2) for the less sensitive parameters, such as, bottom sound speed, bottom density, bottom attenuation and water sound speed, when the SNR is low, the marginal PPD solution can better smooth the noise, which leads to better performance than the MAP solution; (3) for the medium sensitive parameters, such as, source depths, array tilt, and water sound speed, with the change of SNR, the location accuracy of the two methods varies. In summary, the MAP method slightly out-performed the marginal PPD approach in source localization in an unknown environment. So if there is no need to provide uncertainty analysis, the MAP solution is a time saving and high accuracy method.

Fig. 6. The root-mean-square error of the MAP solution and the marginal PPD solution. Both approaches were applied to 50 realizations of noisy acoustic data.

References
Submarine groundwater discharge around Taiwan

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Abstract

A preliminary study shows that the submarine groundwater discharge (SGD) exists around Taiwan even though groundwater overdrawing on the island is serious. Fifteen of the 20 sites studied for major anions and cations recorded a clear SGD signal with freshwater outflow. A total of 278 salinity and major ion measurements were made. Sixteen nearly freshwater SGD (salinity < 1.0) samples were obtained, providing strong and direct evidence for the existence of fresh meteoric groundwater entering the ocean from Taiwan. The total SGD flux is estimated to be 1.07×10¹⁰ t/a which is about 14% of the annual river output. The freshwater component of the SGD is 3.85×10⁸ t which is about 5.2% of the annual river discharge in Taiwan. The collected SGD has a composition similar to seawater with an addition of Ca, CO₃ and HCO₃ due to dissolution of calcareous rocks. Some samples with high Cl/(Na+K) may indicate pollution.

Key words: submarine groundwater discharge, Taiwan, flux, major components, seawater intrusion


1 Introduction

The coastal zone is marked by rich biodiversity and is of great importance for fisheries, agriculture and human settlements. For islands, the groundwater is a particularly important freshwater resource (Aris et al., 2013). Yet, the groundwater in coastal zones faces many threats; of which, seawater intrusion is a notable example. In order to deal with potential threats to this critical resource it is therefore important to understand the freshwater-seawater interface. The seawater intrusion mentioned above involves the penetration of seawater landward. On the other hand, the submarine groundwater discharge (SGD) involves the flow of groundwater and associated dissolved material seaward (the conceptual model is depicted in Fig. 1 of Moore (2010)). SGD, in a way, is a waste of the freshwater resource, and is the subject of this study.

The subtropical island of Taiwan has an area of 35 873 km² which is mostly mountainous with abundant rainfall which averages 2 515 mm or 90×10⁷ m³/a. The alluvial plains with elevation ≤1.0 m cover an area of just 3.7%. The mountainous regions store about 125×10⁹–165×10⁹ m³ of groundwater while the alluvial plains store about 45×10⁹–58×10⁹ m³ groundwater. The annual groundwater recharge is about 4×10⁸–5×10⁹ m³ yet the pumping of groundwater is as much as 7×10⁹ m³/a. In some areas of Taiwan, seawater intrusion has occurred due to the over-pumping of groundwater (Central Geological Survey (CGS), 2002; Peng et al., 2008; Chiang et al., 2013). Nevertheless, Chen et al. (2005) obtained fresh meteoric groundwater with a seepage meter at a water depth of 7.8 m off Southwest Taiwan. Based on a hydraulic model with the help of salinity, deuterium and oxygen-18, Peng et al. (2008) came to the conclusion that, although some coastal areas in Taiwan are experiencing seawater intrusion, some coastal plains still show a surplus of groundwater moving downstream. Lin et al. (2010) and Zavialov et al. (2012) also found some evidence of SGD off Southwest Taiwan based on oceanic chemistry data. The total SGD flux for Taiwan, however, is not known.

The definition of SGD is any and all flow of water on continental margins from the seabed to the coastal ocean, regardless of fluid composition or driving force (Burnett et al., 2003). Basically, SGD is composed of the terrestrial freshwater and circulated seawater driven by various forces (e.g., density, tides and waves). Thus SGD occurs at land-ocean interface at every moment. Here the total flux of SGD around Taiwan is estimated for the first time. Since part of the aim is to study the components of the SGD we relied on the use of seepage meters to collect samples for further analyses.

2 Methods

Preliminary sampling of the SGD was performed from 2004 to 2016. Twenty sampling sites around the coasts of Taiwan are...
shown in Fig. 1. SGD samples for chemical analysis were drawn by a device designed by Zhang and Satake (2003), and either a Lee (1977) type or a conductivity based (Peng et al., 2008) seepage meter (Fig. 2) was used to measure the SGD flux at various tidal ranges. Preserved samples, with saturated HgCl$_2$ added except for salinity and Cl samples, were brought back and Ca, Mg, K, Na, total alkalinity (TA), SO$_4^{2-}$ and Cl were measured in the laboratory following the methods described in Chen et al. (2008). A total of 278 salinity and major ion measurements were made.

3 Results and discussion

The salinity of 278 measurements varies over a wide range between 0.008 and 34.8 with an average of 21.92±11.43, reflecting seawater intrusion. Other ions also show a wide range. Of note is that 16 samples from five sites showed fresh (salinity ≤ 1.0) SGD. The SGD with a salinity of 0.2 was found at a distance of 350 m and a water depth of 7.8 m off Southwest Taiwan. This is where Lin et al. (2010) and Zavialov et al. (2012) found low salinity seawater based on their measurements in the water column.

The average concentrations of the parameters measured are given in Table 1. Out of the 20 sampling sites, 15 showed evidence of some SGD. Five sites without the evidence of SGD are in areas known to be overpumping groundwater. Na and Cl are the dominating cation and anion, respectively (Fig. 3), followed by

![Table 1. Concentrations of major ions measured in the SGD](image)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range</th>
<th>Mean±SD</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salinity</td>
<td>0.008–34.8</td>
<td>21.92±11.43</td>
<td>278</td>
</tr>
<tr>
<td>Ca$^{2+}$/mmol·L$^{-1}$</td>
<td>0.028–36.8</td>
<td>7.98±5.04</td>
<td>116</td>
</tr>
<tr>
<td>Mg$^{2+}$/mmol·L$^{-1}$</td>
<td>0.051–62.5</td>
<td>32.5±16.50</td>
<td>116</td>
</tr>
<tr>
<td>K$^+$/mmol·L$^{-1}$</td>
<td>0.034–10.0</td>
<td>6.13±3.09</td>
<td>116</td>
</tr>
<tr>
<td>Na$^+$/mmol·L$^{-1}$</td>
<td>0.721–464</td>
<td>287±144</td>
<td>116</td>
</tr>
<tr>
<td>HCO$_3^-$/mmol·L$^{-1}$</td>
<td>0.54–8.25</td>
<td>3.00±1.34</td>
<td>123</td>
</tr>
<tr>
<td>CO$_3^{2-}$/mmol·L$^{-1}$</td>
<td>0.007–0.796</td>
<td>0.15±0.09</td>
<td>123</td>
</tr>
<tr>
<td>Cl$^-$/mmol·L$^{-1}$</td>
<td>7.814–623</td>
<td>364±177</td>
<td>100</td>
</tr>
<tr>
<td>SO$_4^{2-}$/mmol·L$^{-1}$</td>
<td>0.206–29.6</td>
<td>36.6±7.17</td>
<td>100</td>
</tr>
</tbody>
</table>

![Fig. 3. Composition of major cations and anions.](image)

![Fig. 4. Piper plot of SGD samples.](image)
Mg and SO$_4$. This is a clear initial indication that seawater is the major component of the SGD either coming directly through submarine intrusion or indirectly through sea spray with subsequent percolation into the groundwater. The Piper plot (Fig. 4) also reveals that the SGD is dominated by the Na(+K)-Cl type (seawater components) with small components of Ca-Cl (mixed seawater and freshwater components) and Ca-HCO$_3$(+CO$_3$) (freshwater components) types. This reflects the influence of seawater and the dissolution of calcareous rocks.

Of note is that many Ca, Mg, SO$_4$, and Cl values are clearly above the seawater slope. The higher Ca and Mg values are perhaps due to the dissolution of calcareous rocks or dolomite. The higher TA values at lower salinities support this suggestion. The higher SO$_4$ and Cl concentrations are, however, likely due to pollution to be discussed below.

The Mg/Ca ratio is shown in Fig. 6a. Most data follow the seawater ratio but some samples show an excess amount of Ca relative to Mg, suggesting dissolution of calcareous rocks. Ratios of Mg/Ca that exceeds 1.0 indicate that the dolomitization process may take place due to the presence of seawater in the groundwater (Pulido-Leboeuf, 2004). In the case for Taiwan, most SGD samples show a seawater Mg/Ca ratio of 5.14, with only a few having an Mg/Ca ratio below 1.0. The Na/Ca ratio (Fig. 6b) does not show a clear pattern except that the values fall around the seawater ratio. The Cl/HCO$_3$ ratio increases with Cl (Fig. 6c). The SO$_4$/Cl values mostly follow the seawater ratio (Fig. 6d) but there are some high values perhaps reflecting pollution. Figure 6e plots the non-sea salt SO$_4$ (nss-SO$_4$)/(Na+K) vs. Cl/(Na+K). The values above the seawater ratio reflect pollution of Cl from garbage incineration or petrochemical plants.

Altogether there are only 44 flux measurements with an average of (1.67±0.7) L/(m$^2$·h) for SGD and (0.37±0.47) L/(m$^2$·h) for the freshwater outflow. These small sample numbers are not sufficient to obtain a robust average because of the large seasonal and spatial variations. Besides, fluxes must be heavily influenced by tidal phases due to different sea level responses (Liu et al., 2018). Since we measured the fluxes at various tidal phases the total SGD flux to be given below is subject to large uncertainties.

Assuming that the SGD exists in a 1 km wide band around Taiwan with a 1 200 km long coast line the first approximation of the SGD export results in a value of (1.07±0.7)×10$^{10}$ t/a; of which, (0.38±0.48)×10$^{10}$ t/a is the freshwater component. These values are, respectively, about 14% and 5.2% of the total river outflow from Taiwan, and fall within the ranges reported elsewhere (Zektser et al., 1983; Church, 1996; Moore, 1996; Cable et al., 1996; Burnett et al., 2001, 2003; Taniguchi et al., 2002, 2008). More specifically, Moosdorf et al. (2015) estimated the global average fresh groundwater discharge at 7 050 m$^3$/a with Taiwan having a value of 5 486 m$^3$/a. Our freshwater SGD component translates to (3 200±4 000) m$^3$/a. Considering the large uncertainty, the agreement is reasonable.

Across the Taiwan Strait the SGD has also been studied in the Jiulong River with its 14 700 km$^2$ watershed falling in the same latitude as Taiwan (Fig. 1). The recent work of Wang et al. (2015) reported an SGD value in the Jiulong River Estuary as 8%–19% of the concomitant river discharge, compared with 14% obtained above for Taiwan (Fig. 1). In terms of the freshwater component of the SGD the Jiulong River Estuary exports about 2%–4.8% of the concomitant river discharge, compared to 5.2% for Taiwan. Considering the large uncertainties involved, these values can be considered similar.
4 Conclusions

Two hundred and seventy-eight salinity and major ion measurements were performed on the SGD collected at 20 sites around Taiwan. Fifteen sites showed evidence of some freshwater outflow. The collected SGD has a composition similar to seawater with an addition of Ca, CO$_3$ and HCO$_3$ due to dissolution of calcareous rocks. Some samples with high Cl/(Na+K) may indicate pollution. Forty-four flux measurements reveal that the total flux of SGD from Taiwan amounts to 14% of the total river outflow.

References


Reducing eutrophication risk of a reservoir by water replacement: a case study of the Qingcaosha reservoir in the Changjiang Estuary

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Abstract
Eutrophication of freshwater systems in cities is a major concern worldwide. Physical, biological and chemical methods have been used in eutrophic lakes and reservoirs to reduce their eutrophic state and algal biomass, but these approaches are not effective without a substantial reduction in nutrients input, which could take decades to achieve in the developing countries. This study aims to assess the risk of eutrophication and algal bloom in a coastal reservoir with high nutrient inputs to confirm the feasibility of inhibiting the reservoir’s eutrophic state by hydrodynamic operations. A variety of water quality indexes (e.g., water temperature, secchi depth, dissolved oxygen, total nitrogen, total phosphorus, phytoplankton chlorophyll a) at five observed sites were investigated in the Qingcaosha reservoir, which located in the Changjiang Estuary, during the construction, trial and normal operation periods from 2009 to 2012. No water exchange happened during the construction from April 2009 to October 2010, and the water exchange increased during the trial from October 2010 to January 2011, and during normal operation period from January 2011. The comprehensive nutrition state index (TLI) calculated by several representative water quality indexes was adopted to evaluate the variation of the trophic state in the reservoir. The peak values of TLI reached 51 in the summer of 2009, and 55 in the summer of 2011, higher than the eutrophication threshold value 50. The lowest TLI, about 32, appeared in the summer of 2010. The values of TLI in other observation periods could keep under 50. The results showed that the reservoir could easily deteriorate into the eutrophic state because of excess nutrients and algal blooms in the summer of 2009 and 2011, while the eutrophication and algal blooms could be reduced by the lack of nutrients in 2010 or adequate water replacement in 2012. The temporal and spatial variations of water quality indexes were presented based on observation data and analysis. The adequate water replacement in the reservoir driven by tides was tested to be an efficient and economical method for controlling eutrophication and algal blooms in the water environment with high nutrient inputs.

Key words: estuarine reservoir, eutrophic state, algal bloom, operation way


1 Introduction
Aquatic systems impacted by human activities continue to deteriorate with the economic development. Because of the high input of nutrients, many freshwater systems have become eutrophic, and the resulting algae blooms have triggered drinking water crises throughout the world (Carmichael, 2001; Huisman et al., 2005; Paepl et al., 2001). Lake Erie (US/Canada), Lake Winnipeg (Canada), Lake Victoria (the largest of the African rift lakes), and Lakes Biwa and Kasimagaura (Japan’s largest lakes) have suffered from eutrophication (Paepl et al., 2011). Many lakes and reservoirs in China, especially those near urban areas, have deteriorated to a eutrophic state due to excessive inflows of nitrogen, phosphorus, organic matter and other pollutants (Chen et al., 2009). The Chinese government and many researchers have adopted measures to recover and manage these eutrophic lakes. Restoration projects have been implemented at the Lake Taihu (Pu et al., 1993), Lake Dianchi (Li et al., 2005), Lake Wuli (Chen et al., 2006), Lake Mochou (Pu et al., 2001), and other small lakes and reservoirs (Tu et al., 2004) throughout China.

As the financial center of China, Shanghai is home to more than twenty million residents. Poor water quality has troubled this city for many years. In 2007, a coastal reservoir project was initiated in the Changjiang Estuary to build the largest drinking water source in Shanghai. This estuarine reservoir, named the Qingcaosha Reservoir, was designed to supply 7,190,000 m³ of freshwater per day (more than 50% of the total freshwater supply in Shanghai) and serve more than 13 million people in Shanghai. It was finished in 2010 and started normal operation in 2011. It is located on the northern end of Changxing Island (Fig. 1). The distribution of freshwater and saltwater fluctuates due to tidal activity in this area (Chang et al., 2014; Qiu and Zhu, 2013; Wu et al., 2006). Utilizing the difference of water level between inside and outside of the reservoir, the water gates at the northwest (upper reach) and southeast (lower reach) of the Qingcaosha Reservoir...
are manually controlled to draw and discharge freshwater and to avoid saltwater when replacing water in the reservoir. The total area of the reservoir is 66.15 km$^2$. The water depth in the reservoir varies from 2.7 m to 12.1 m, generally increasing from the northwest (upper reach) to the southeast (lower reach). A large central wetland was set in the northwest central position of the reservoir. This reservoir was enclosed and had no water exchange with the Changjiang Estuary while under construction from April 2009 to September 2010. The project was completed and began to draw water in October 2010. The water supply of the reservoir was approximately 800 000 t/d and the water gates opened only once per day to draw water from the Changjiang Estuary during the trial period from October 2010 to December 2010. After the trial period, the water supply gradually increased to $2.5 \times 10^6$ t/d at the end of 2011 and $4 \times 10^6$ t/d at the end of 2012. The water gate operation was changed to run twice per day to take water in the reservoir by means of the semi-diurnal tides.

The substantial water discharge of the Changjiang River exports abundant nutrients into the estuary. The annual nutrients flowing to the sea by the Changjiang River is markedly higher than in other estuaries in China (Shen et al., 1992). Its annual flows of total inorganic nitrogen, phosphate, silicate and nitrate are $8.88 \times 10^6$ t, $1.36 \times 10^4$ t, $2.04 \times 10^5$ t and $6.36 \times 10^6$ t, respectively (Gao and Song, 2005). Sharp reduction of such high nutrient inputs for the Qingcaosha Reservoir is difficult and expensive. The researchers and managers of the Qingcaosha Reservoir want to find some efficient and economical methods to keep the trophic state of the drinking water source at a safe level. This study aims to assess the risk of eutrophication and algal blooms in the estuarine reservoir with high nutrient inputs to confirm the feasibility of inhibiting the reservoir’s eutrophic state by appropriate reservoir hydraulic operations.

2 Methods

Five observation sites were designed in the Qingcaosha Reservoir (Fig. 1). Site #1 was located in the northwest of the reservoir; Sites #2 and #3 were located at the southern and northern ends of the central wetland, respectively; Site #4 was located near the center of the reservoir; and Site #5 was located at the southeast end of the reservoir. The depths of Sites #1-#5 are 2.7 m, 4.6 m, 8.6 m, 9.7 m, and 10.8 m, respectively. The investigation began in September 2009 at Site #1 and began in April 2009 at the other sites. The monitoring work was completed in December 2012. The water was sampled at a depth of 0.5 m below the surface and 0.5 m above the bottom at each site. Water temperature, Secchi depth (SD) and dissolved oxygen (DO) were measured at all sites. Chemical oxygen demand (COD$_{Mn}$), ammonia nitrogen (NH$_3$-N), nitrate nitrogen (NO$_3$-N), nitrite nitrogen (NO$_2$-N), total nitrogen (TN), total phosphorus (TP), dissolved total phosphorus (DTP), and phytoplankton chlorophyll a (Chl a) were sampled at all sites and analyzed in the laboratory. The sampling, measurement and analysis methods all followed the standard procedures recommended by the Ministry of Environmental Protection of the People’s Republic of China (Wei, 2002) (Table 1).

The comprehensive nutrition state index (TLI) was adopted to evaluate the trophic state of the Qingcaosha Reservoir. Five water quality indexes (Chl a, TP, TN, SD and COD$_{Mn}$) were selected to calculate the TLI (Jin and Tu, 1990). The trophic states of lakes and reservoirs are classified into different levels according the TLI values (Table 2), which can range from 0 to 100.

The equation to calculate the TLI is as follows:

![Fig. 1. Map of the Changjiang Estuary and the main fresh water resources near Shanghai. The study region is the Qingcaosha Reservoir which located on the northern end of Changxing Island. The black dots with numbers in the reservoir denote the observation sites.](image-url)
Table 1. Water quality index analysis methods (Wei, 2002)

<table>
<thead>
<tr>
<th>Index type</th>
<th>Index name</th>
<th>Analysis method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical index</td>
<td>water temperature</td>
<td>thermometer method</td>
</tr>
<tr>
<td></td>
<td>dissolved oxygen</td>
<td>electrochemical probe method</td>
</tr>
<tr>
<td></td>
<td>Secchi depth</td>
<td>Secchi disc method</td>
</tr>
<tr>
<td>Nutrient index</td>
<td>ammonia nitrogen</td>
<td>Nessler’s reagent spectrophotometry</td>
</tr>
<tr>
<td></td>
<td>nitrate nitrogen, nitrite nitrogen</td>
<td>phenol disulfonic acid spectrophotometry molecular absorption spectrophotometry</td>
</tr>
<tr>
<td></td>
<td>total nitrogen</td>
<td>alkaline potassium persulfate digestion-UV spectrophotometry</td>
</tr>
<tr>
<td></td>
<td>total phosphorus</td>
<td>ammonium molybdate spectrophotometry</td>
</tr>
<tr>
<td></td>
<td>dissolved total phosphorus</td>
<td>ammonium molybdate spectrophotometry</td>
</tr>
<tr>
<td></td>
<td>chemical oxygen demand</td>
<td>potassium permanganate index method</td>
</tr>
<tr>
<td>Phytoplankton index</td>
<td>Chl a</td>
<td>spectrophotometric method</td>
</tr>
</tbody>
</table>

Table 2. Water trophic state classification according to TLI (Jin and Tu, 1990)

<table>
<thead>
<tr>
<th>Trophic state</th>
<th>TLI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligotrophic</td>
<td>TLI&lt;30</td>
</tr>
<tr>
<td>Mesotrophic</td>
<td>30≤TLI≤50</td>
</tr>
<tr>
<td>Eutrophic</td>
<td>mild eutrophic state: 50&lt;TLI≤60</td>
</tr>
<tr>
<td></td>
<td>medium eutrophic state: 60&lt;TLI≤70</td>
</tr>
<tr>
<td></td>
<td>hyper-eutrophic state: TLI&gt;70</td>
</tr>
</tbody>
</table>

\[ TLI = \sum_{j=1}^{m} W_j \times TLI_j, \quad (1) \]

where \( m \) is the number of water quality indexes, \( j \) is the number of each water quality index, \( W_j \) is the weight of each water quality index, and \( TLI_j \) is the calculated TLI of each water quality index. Based on the Chl \( a \) value, the value of \( W_j \) is calculated as follows:

\[ W_j = r_{ij}^2 / \sum_{j=1}^{m} r_{ij}^2, \quad (2) \]

where \( r_{ij} \) indicates the correlation coefficient of the water quality index \( j \), calculated according to the reference parameter Chl \( a \). \( r_{ij} \) is determined according to the calculated results from the 26 major lakes in China (Jin, 1995) (Table 3).

Table 3. The \( r_{ij} \) of water quality indexes (Jin and Tu, 1990)

<table>
<thead>
<tr>
<th>Correlation coefficient</th>
<th>Chl ( a )</th>
<th>TP</th>
<th>TN</th>
<th>SD</th>
<th>COD(_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>( r_{ij} )</td>
<td>1</td>
<td>0.84</td>
<td>0.82</td>
<td>0.83</td>
<td>0.83</td>
</tr>
</tbody>
</table>

The TLI of each water quality index is calculated as follows:

\[ TLI (Chl \ a) = 10 \times (2.5 + 1.086 \times \ln \text{Chl} \ a), \quad (3) \]
\[ TLI (TP) = 10 \times (9.436 + 1.624 \times \ln \text{TP}), \quad (4) \]
\[ TLI (TN) = 10 \times (5.453 + 1.694 \times \ln \text{TN}), \quad (5) \]
\[ TLI (SD) = 10 \times (5.118 + 1.94 \times \ln \text{SD}), \quad (6) \]
\[ TLI (COD\(_{50}\)) = 10 \times (0.109 + 2.66 \times \ln \text{COD}\(_{50}\)). \quad (7) \]

3.1 Water quality index analysis in the Qingcaosha Reservoir

There was little spatial variation of water temperature in the reservoir during the observation period. No thermocline was observed in this reservoir. The water temperature in the entire reservoir fluctuated seasonally, ranging from 2.5°C in winter to 32.5°C in summer.

When the reservoir was enclosed, the vertical DO concentrations varied greatly at the measured sites due to the deficiency of water exchange and the influence of phytoplankton photosynthesis at the surface layer. The maximum difference of DO between the surface layer and bottom layer was 6 g/m³. This phenomenon disappeared after the reservoir began operations. DO also fluctuated seasonally, ranging from 6 g/m³ in summer to 14 g/m³ in winter.

The SD in the reservoir was mainly influenced by the growth of phytoplankton and wave induced by wind. The SD generally decreased in summer and increased in winter and spring. The peak value of SD at Site #5 was 1.8 m in the spring of 2010, 1.6 m in the spring of 2011 and 1.7 m in the winter of 2011. The valley value at Site #1 was 0.5 m in the summer of 2009 and 0.17 m in the summer of 2010. With the increasing water exchange in the reservoir, the SD at Site #1 gradually stabilized at 0.25 m. However, obvious fluctuations of SD continued at the other observation sites (Fig. 2). The horizontal variation of SD was primarily increasing from the northwest (the upper reach) to the southeast (the lower reach). This phenomenon could be explained by the settlement process of the suspended solids (SS). The SS was drawn from the northwest gate and kept settling with the water movement to the southeast end of the reservoir.

Based on the annual averaged data at the observation sites, the dissolved inorganic nitrogen (DIN), considered as the sum of NH\(_4\)-N, NO\(_2\)-N and NO\(_3\)-N, accounted for 63.1%–67.8% of the TN. The proportion of particulate nitrogen (PN) in the TN was low. Thus, the TN was not mainly influenced by the wave and SS, and varied little in the vertical during the observation period (Fig. 3). The spatial difference of TN was small during the reservoir’s closed period, except that the TN at Site #1 was slightly higher than at the other sites in the summer of 2009 and the spring of 2010, because of the growth of phytoplankton. The concentration of TN in the entire reservoir decreased from 1.8 g/m³ to 0.5 g/m³ during the closed period due to the lack of nutrients input and water self-purification (Fig. 2). The concentration of TN at Site #1 clearly increased after October 2010 because of the high nutrient inputs from the Changjiang Estuary. It exceeded 2.6 g/m³ in July 2011 and fluctuated in the range of 1.2–2.2 g/m³ in the remaining observation period. During the operation period, the concentration of TN at Site #1 was substantially higher than the other sites, and the others did not obviously differ from each other (Fig. 2). This showed that the TN decreased mainly in the...
northwest area, because of the nutrients absorption function of the central wetland. Wetlands are well known as the highly effective systems mitigating the negative effects of nitrogen and phosphorus excess. Natural and artificial wetlands are widely applied in the worldwide (Alongi, 2008; Álvarez-Rogel et al., 2016; Kang et al., 2017).

The major component of TP in the reservoir was particulate phosphorus (PP). The annual averaged ratios of DTP/TP were

![Fig. 2. Time series of SD, TN, TP and Chl a from 2009 to 2012 at the observation sites. The values of TN, TP and Chl a were observed at a depth of 0.5 m below the water surface. Two vertical dotted lines separate the observation period into three sections: closed period, trial period and normal operation period.](image-url)
only 26.2%–30.6% at the measured sites. Therefore, an intense fluctuation was found in the analyzed results of TP. The range of TP was 0.005–0.11 g/m$^3$ during the reservoir’s closed period, and this range changed to 0.01–0.17 g/m$^3$ after the reservoir began to operate (Fig. 2). Due to the settlement of SS, the TP in the surface layer was lower than in the bottom layer from 2009 to 2011. After 2012, the surface concentration of TP was occasionally higher than the bottom concentration (Fig. 3) because of the gradually intensified vertical turbulence during the operation period. The horizontal variation of TP was mainly decreasing from northwest to southeast. Along with the increase of nutrients input, the horizontal gradient of TP gradually increased after the reservoir began to operate in October 2010. This phenomenon was observed in the entire reservoir (Fig. 2), primarily due to the nutrients absorption function of the central wetland and the settlement of PP in the entire reservoir.

The surface concentration of Chl $\alpha$ was much higher than the bottom concentration in the summers of 2009 and 2011, because of the rapid growth of phytoplankton. However, the bottom concentration of Chl $\alpha$ was occasionally higher than the surface concentration in 2012 due to the gradually intensified vertical turbulence (Fig. 3). For the stagnant water environment and sufficient nutrients, the phytoplankton grew quickly throughout the reservoir in 2009. An algal bloom occurred and the peak concentration of Chl $\alpha$ at Site #1 reached 50 mg/m$^3$ in the summer of 2009 (Fig. 2). The concentrations of TN and TP decreased to 0.5 g/m$^3$ and 0.01 g/m$^3$ in the summer of 2010 because of no nutrient inputs (the reservoir was enclosed) and water self-purification. The Qingcaoasha Reservoir had not reached a eutrophic state (Nürnberg, 1996), and the low nutrients could not support the growth of phytoplankton. Therefore, the concentration of Chl $\alpha$ was reduced to 2 mg/m$^3$ and no algal bloom happened in the
summer of 2010. After October 2010, the reservoir began to operate, and the fresh water with high nutrients in the Changjiang Estuary was drawn into the reservoir. The growth of phytoplankton rose again and the peak concentration of Chl \(\alpha\) at Site #1 reached 25 mg/m\(^3\) in the summer of 2011. However, the growth of phytoplankton had been weakened with the gradual increase of water replacement in the reservoir since 2012. The Chl \(\alpha\) in the entire reservoir was limited to less than 10 mg/m\(^3\) in the summer of 2012, even with high concentrations of nutrients. The variations of Chl \(\alpha\) in 2012 show that the adequate water replacement is an effective method for controlling algae blooms in a eutrophic water environment. The similar conclusion was reached in the study of Dianshan Lake in Shanghai, the accumulation and growth of phytoplankton in lakes and reservoirs with high nutrients could be effectively inhibited by the increasing discharges (Chen et al., 2016). The main reason of this phenomenon is that the algae can be quickly transported out of lakes and reservoirs by adding inflow and outflow discharges before they have the opportunity to bloom.

3.2 Trophic state assessment of the water environment in the Qingcaosh Reservoir

Using the spatially averaged data, the TLI was calculated to reflect the variation of the water trophic state in the Qingcaosh Reservoir (Fig. 4). The trophic state of the reservoir deteriorated from mesotrophic state to mild eutrophic state because of the rapid growth of phytoplankton, sufficient nutrients and the obvious decline of SD from July 2009 to October 2009. The trophic state quickly recovered to mesotrophic state due to the low concentrations of nutrients and the slow growth of phytoplankton from November 2009 to September 2010. The reservoir began to operate in October 2010, and the growth of phytoplankton was then reactivated along with the high nutrient inputs from the Changjiang Estuary. Thus, the trophic state of the Qingcaosh Reservoir gradually deteriorated to a mild eutrophic state again in July 2011. With regard to the increase of TN and TP, the peak value of TLI in the summer of 2011 was higher than in the summer of 2009. In 2012, the concentrations of nutrients in the reservoir remained high due to the increasing inflow from the Changjiang Estuary, but the growth of phytoplankton was restricted because of the more frequent water replacement (Fig. 2). Thus, the trophic state of the Qingcaosh Reservoir remained mesotrophic in 2012 (Fig. 4).

According the analysis above, the nutrients in the Qingcaosh Reservoir, which is the cause of the eutrophication and algal blooms, are difficult to reduce because the nutrients input from the Changjiang Estuary is high. Reducing the input of nutrients is still the ultimate method to address eutrophication in lakes and reservoirs (Edmondson, 1970; Schindler, 2006), but this could take decades to achieve in the developing countries (Paerl et al., 2011; Qin et al., 2015). The toxins and odorous compounds generated by algae blooms are much more dangerous than normal nutrients in a drinking water system (Burgos et al., 2014; Ma et al., 2013; Song et al., 2007; Ueno et al., 1996), and the technological requirements and costs required to remove the former are much higher than the latter. Biological methods such as filter-feeding fish, shellfish and aquatic plants, and physical methods including mechanical measures and artificial isolation equipment have been used in eutrophic lakes to reduce algal biomass, but these approaches are not effective without a substantial reduction in nutrients input (Chen et al., 2009; Pu et al., 1993). The positive effects of artificial water diversion and exchange have been proven to decrease the concentrations of phytoplankton in non-tidal areas, but it is difficult to exchange water sufficiently over an entire area and the cost is prohibitive (Hu et al., 2008; Li et al., 2013).

![Fig. 4. Time series of TLI calculated with the spatially averaged observation data from 2009 to 2012 in the Qingcaosh Reservoir. The horizontal dotted line represents the threshold of eutrophic state according to TLI.](image-url)

The results of this study show that the adequate water replacement driven by tides is an appropriate operation way to inhibit the eutrophic state of estuarine reservoirs with high nutrient inputs. Reservoirs set in estuarine area have a natural advantage in restricting the growth of phytoplankton in the high nutrients environment by utilizing the tidal range, and these reservoirs can exchange water frequently with the control of water gates. It is an efficient and economical way to low down the risk of eutrophication and algal blooms in the drinking water sources, while it takes decades to reduce the input of nutrients loads from the upper reaches to a safe level.

4 Conclusions

The variations of the nutrients and Chl \(\alpha\) in the Qingcaosh Reservoir were analyzed based on the observed data at five sites from 2009 to 2012. In the summer of 2009, the unfinished reser-
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On the sediment age estimated by 210Pb dating: probably misleading “prolonging” and multiple-factor-caused “loss”

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Abstract

The radionuclide 210Pb is suitable for century-scale dating and has been used to calculate the sedimentation rate in a variety of environments. However, two common ways to apply 210Pb dating techniques may give misleading results. One is “prolonging of age”, i.e., using the calculated sedimentation rate to date back to 200 or 300 years. This practice must be treated with caution because the 210Pb dating techniques do not guarantee direct dating for ages much older than 100 years. Another is “loss of age”, i.e., the calculated time span between the topmost layer and the 210Pb background layer in cores is less than 100 years when an apparent sedimentation rate is used in the calculation. Here, we propose that based on the principle of 210Pb dating, the upper limit of age suitable for direct 210Pb dating is between 110 and 155 years. The “prolonging” application is acceptable only if the sedimentary environment in the past several hundred years was stable and the sedimentation rate was generally constant, and verification with independent evidence (such as historical records or biomarker methodology) is needed. Furthermore, after analyzing many published and collected data, we found four possible reasons for the "loss of age". First, the compaction effect of sediment should be considered in laboratory analysis or else the calculated age will be underestimated. Second, the accuracy and uncertainty of 210Pb activity measurement affect the judgment of the background. To be cautious, researchers are apt to choose a background activity with a younger age. Third, use of a slightly smaller value of supported 210Pb activity in a calculation will lead to considerable underestimation of the sedimentary records and are often reflected as a "loss of age" in cores. We believe that proper use of 210Pb dating data may provide helpful information on our understanding of sediment records and recent environmental changes.

Key words: 210Pb dating, sedimentation rate, sediment flux


1 Introduction

The natural radionuclide 210Pb, an intermediate daughter of the 238U decay series, has a half-time of 22.26 years and is obtained from the 3α decay, 2β decay and 1α decay of 226Ra (half-time of 1602 years) (Fig. 1). The daughter of the first α decay is the noble gas element 222Rn, whose half-time is only 3.82 d (Liu, 2010). The 210Pb in sediments have two major sources, unsupported and supported. The unsupported 210Pb primarily originated from atmospheric deposition (including wet and dry deposition, as the decay daughter of 222Rn), and it is then absorbed by suspended particulates and eventually enters sediment. This fraction of 210Pb is also called “excess 210Pb” and denoted 210Pb insane. The supported 210Pb is the decay daughter of 226Ra in sediments and is denoted 210Pb eq. Generally, if disturbance, erosion and diffusion in sediments are ignored, the 210Pb insane that enters sediments will no longer receive atmospheric supply and will follow the general decay law of radionuclides, i.e., its activity decreases by half every 22.26 years. While the 210Pb insane and precursor 226Ra follow the long-term equilibrium relation of radioactive decay, the change in 210Pb eq. within 100 years is negligible. When the above conditions are satisfied, we can infer the age of the layers by measuring the radioactivity of 210Pb eq. at different layers in short cores. Combining this result with the depth and density of layers, we can calculate the sedimentation rate (unit: g/(cm²·a)) or apparent deposition rate (unit: cm/a).

Ever since Goldberg (1963) proposed the principle of 210Pb dating, geologists have widely applied the principle to dating and sedimentation rate calculations for ice cores, soils, lakes, estuaries, tidal flats, lagoons, bays and inner shelves (Koide et al., 1972; Nitttrouer et al., 1979; Zou et al., 1982; Appleby and Oldfield, 1983; Qian et al., 1985; DeMaster et al., 1985; Alexander et al., 1991; Li et al., 1996; Wan, 1997; Xia et al., 1999, 2004; Chen et al., 2004; Zhang et al., 2009; Liu et al., 2009; Jia et al., 2012). It has become a powerful tool for studying century-scale sedimentary records and
environment evolution. However, Binford (1990) once remarked, nearly 30 years after the technique of $^{210}$Pb dating was proposed, that "$^{210}$Pb-dating are described mathematically in numerous papers, but actual calculation methods are never explicit. Estimates of dating uncertainty are seldom presented in published papers or reports". Even today, this comment can still cause resonance among researchers.

Based on a literature review, we found two phenomena in the applications of $^{210}$Pb dating techniques. One is "prolonging", i.e., the dating age is backtracked using the calculated sedimentation rate; the backtracking period can reach 200 or 300 years in certain cases. The other is "loss of age", i.e., the period above the $^{210}$Pb background layer is calculated according to the measured apparent deposition rate and is less than 100 years in many cases. Both phenomena have certain problems. The application of "prolonging" already exceeds the time scale ensured by the long-term equilibrium between the precursor $^{226}$Ra and daughter $^{210}$Pb. The "loss of age" phenomenon may be due to several problems or errors that occur during laboratory pretreatment of samples, interpretation of data measured by an energy spectrometer or calculation procedures adopted for determination of ages, or it may indicate disturbances or missing layers in sedimentary records. However, for a long time, the former was applied thoughtlessly, and the latter was presented blindly.

Based on the principle of $^{210}$Pb dating, the "prolonging" and "loss of age" phenomena are discussed in this study with collected $^{210}$Pb data of short cores. We assessed the suitability and reliability of the former and examined the causes of the latter and precautions in interpretation. The objective of this paper is to promote a better understanding of $^{210}$Pb dating data.

### 2 Principle of $^{210}$Pb dating and sedimentation rate calculation

Two of the most common mathematical models for $^{210}$Pb dating (Oldfield and Appleby, 1984) are the constant initial concentration (CIC) model and the constant rate of supply (CRS) model. More $^{210}$Pb dating models are described by Wan (1997), Zhang et al. (2008) and Sanchez-Cabeza and Ruiz-Fernández (2012).

#### 2.1 The CIC model

The basic assumption of the CIC model is that the suspended particles in water absorb $^{210}$Pb proportionally. When the suspended particle flux deposited into the water-sediment interface increases, the absorbed $^{210}$Pb flux that enters the water-sediment interface also increases. In other words, regardless of how the accumulation rate of sediment changes at the water-sediment interface, the $^{210}$Pb concentration at the water-sediment interface is constant. Therefore, the variation in the $^{210}$Pb concentration in the sediments with depth follows the following equation (Appleby and Oldfield, 1983):

$$C = C_0 e^{-\lambda t},$$

where $C_0$ is the initial $^{210}$Pb$_{ac}$ activity (dpm/g) at the water-sediment interface, $C$ is the $^{210}$Pb$_{ac}$ activity (dpm/g) at a layer in the sediments, $\lambda$ is the $^{210}$Pb decay constant ($3.114 \times 10^{-2}$ a$^{-1}$), and $t$ is the layer age (a) corresponding to $C$, which can be calculated according to the following equation:

$$t = \frac{1}{\lambda} \ln \frac{C_0}{C}.$$
fy the following conditions (Appleby and Oldfield, 1983).

1. The variation in the $^{210}\text{Pb}_{\text{eq}}$ concentration with increasing depth should be monotonic and always decreasing.
2. The differences in the accumulative $^{210}\text{Pb}_{\text{eq}}$ flux among different cores from the same sedimentary environment (such as a lake) should be approximately proportional to the difference in the sedimentation rate.

### 2.2 CRS model

The basic assumption of the CRS model is that for certain local regions, the deposition flux of atmospheric $^{210}\text{Pb}_{\text{eq}}$ entering the water-sediment interface is constant. Because the suspended particles in water absorb $^{210}\text{Pb}_{\text{eq}}$ rapidly and efficiently, the deposition flux of the $^{210}\text{Pb}_{\text{eq}}$ in water entering the water-sediment interface is also constant and not affected by the sediment accumulation rate.

Therefore, the relation between the accumulated $^{210}\text{Pb}_{\text{eq}}$ in sediments and age is expressed by the following equation (Appleby and Oldfield, 1983):

$$A_t = A_0 e^{-\lambda t},$$

where $A_0$ is the accumulative activity of all the $^{210}\text{Pb}_{\text{eq}}$ from the water-sediment interface down to the background, and $A_t$ is the accumulative activity of the $^{210}\text{Pb}_{\text{eq}}$ from a layer in the sediments down to the background. Both can be obtained by integrating the $^{210}\text{Pb}_{\text{eq}}$ activity of the corresponding intervals of cores. Additionally, $t$ is the age of the layer corresponding to $A_t$ and is calculated as follows:

$$t = \frac{1}{\lambda} \ln \frac{A_0}{A_t}.$$  

Sedimentary records suitable for the CRS model should satisfy the following three conditions (Appleby and Oldfield, 1983):

1. The variation curve of the $^{210}\text{Pb}_{\text{eq}}$ activity in sediments may show some fluctuation due to changes in the sediment accumulation rate because an increase in the sedimentation rate can decrease the initial $^{210}\text{Pb}_{\text{eq}}$ activity at the water-sediment interface and vice versa.
2. For different cores taken from the same or similar sedimentary environments (such as from the same lake or certain regions with generally similar sedimentary environment characteristics), although their sedimentation rates may vary, the total deposition flux of $^{210}\text{Pb}_{\text{eq}}$ is generally similar.
3. The total accumulated $^{210}\text{Pb}_{\text{eq}}$ in cores should reflect the atmospheric $^{210}\text{Pb}_{\text{eq}}$ deposition flux in the region.

The mature algorithm of the CRS model was first proposed in 1978 (Appleby and Oldfield, 1978; Robbins, 1978). When it was applied to lacustrine sediments dating, the results matched extremely well with the lamina record of lacustrine sediments. This work was published in *Nature* and drew broad attention (Appleby and Oldfield, 1979).

### 2.3 Sedimentation rate calculation

Suppose the sedimentation rate of sediments is $R$ (g/(cm$^2$-a)); then,

$$t = \frac{M}{R},$$

where $M$ is the mass depth (g/cm$^2$) corresponding to age $t$ (a).

If we use the CIC model to calculate the sedimentation rate, we substitute Eq. (5) in Eq. (1) and then take the logarithm, as follows:

$$\ln C = -M \frac{\lambda}{R} + \ln C_0.$$  

Equation (6) is a one-dimensional linear equation of the form $y=ax+b$, where $y=\ln C$, $x=\lambda t$, $a=-\frac{\lambda}{R}$ and $b=\ln C_0$. We take the logarithm of the $^{210}\text{Pb}_{\text{eq}}$ of each layer in the cores and plot against the corresponding mass depth $M$; then, the sedimentation rate is $R=\frac{\lambda C_0}{b}$.

If we use the CRS model to calculate the sedimentation rate, the deposition flux ($R$, g/(cm$^2$-a)) in a certain layer can be obtained:

$$R = \frac{\lambda A_0}{A_t}.$$  

Subsequently, the apparent sedimentation rate ($r$, cm/a) is expressed as follows:

$$r = \frac{R}{\rho},$$

where $\rho$ (g/cm$^3$) is the bulk density of the sediment. For simplicity, several researchers have assumed that the bulk density of the sediment from the top to the bottom of a core is constant. The apparent sedimentation rate is intuitive; however, the assumption of constant bulk density of the sediment is an important reason for the “loss of age” phenomenon in $^{210}\text{Pb}$ dating. We will further analyze this below.

### 3 The upper limit of age and “prolonging” in $^{210}\text{Pb}$ dating

#### 3.1 The upper limit of age for $^{210}\text{Pb}$ dating

According to the principle introduced in Section 2, the $^{210}\text{Pb}_{\text{eq}}$ variation with time is the key for $^{210}\text{Pb}$ dating and the calculation of sedimentation rates. Thus, we need to know the value of $^{210}\text{Pb}_{\text{eq}}$ (i.e., the $^{210}\text{Pb}$ background). There are generally two procedures to obtain $^{210}\text{Pb}_{\text{eq}}$ (Su et al., 1984). For Procedure I, $^{226}\text{Ra}$ and $^{210}\text{Pb}$ in sediments are assumed to have already reached equilibrium. Thus, we take the $^{226}\text{Ra}$ radioactivity as a constant within a century-scale and then directly measure the precursor $^{226}\text{Ra}$ activity as the background, i.e., $^{226}\text{Ra}_{\text{eq}}$. For Procedure II, we observe the $^{210}\text{Pb}_{\text{eq}}$ variation with depth and generally find a stable value of $^{210}\text{Pb}_{\text{eq}}$ below a certain depth. We take this stable value as $^{210}\text{Pb}_{\text{eq}}$.

Fundamentally, both procedures rely on the long-term equilibrium principle of a radioactive decay series. According to this principle, if the half-time of the precursor is extremely long and if the half-lives of all daughter products are relatively short, the entire decay series reaches long-term equilibrium after a sufficiently long time. A decay series that has reached long-term equilibrium has an important characteristic, i.e., the activities of the precursor nuclides and daughter nuclides are equal, and all nuclides decay following the decay law of the precursor. Therefore, the abovementioned two procedures for determining the $^{210}\text{Pb}$ background measure either the precursor activity or the daughter activity at equilibrium.

Different studies have given slightly different times necessary for reaching long-term equilibrium. Generally, it is five to seven times the longest half-time of the daughters (Cai, 2005; Liu, 2010). From Fig. 1, in the decay series from $^{226}\text{Ra}$ to $^{208}\text{Pb}$, the
daughter with the longest half-time is $^{210}$Pb. Therefore, we can assume that after 110 years (i.e., five times as long as the half-time of $^{210}$Pb) to 155 years (i.e., seven times as long as the half-time of $^{210}$Pb), $^{226}$Ra and its daughter $^{210}$Pb reach long-term equilibrium in sediments. In this regard, it is appropriate to take 155 years as the upper limit of $^{210}$Pb dating. Otherwise, radioactive decay of $^{210}$Pb will follow the law of its precursor $^{226}$Ra, whose half-time is 1602 years. As a consequence, neither Eq. (2) nor Eq. (4) will be appropriate for dating with $^{210}$Pb.

More importantly, the upper limit of $^{210}$Pb dating is related to the lower limit of detection (LLD, unit: Bq) of certain instruments under consideration. Few instruments can distinguish between $^{210}$Pb and the residual activity of $^{210}$Pb if the latter is less than the LLD. According to CNS (1996), the LLD of an $\alpha$ spectrometer can be estimated with the following equation:

$$\text{LLD} \approx 2K S_0,$$

where $S_0$ is the standard deviation of measured activities of samples and $K$ is a statistics parameter depending on the confidence level and tolerance (Table 1). Four cases are illustrated in Fig. 2, and it is clear that after four or five half-lives, the residual activity falls within or even below the LLD in each case. In such situations, the $^{210}$Pb method is unsuccessful at direct dating.

### 3.2 Reliability of the "prolonging" application for $^{210}$Pb dating

As discussed in Section 3.1, a time scale of 100–300 years between the present and the past is generally stable and that the sedimentation rate was generally constant. When these conditions are satisfied, we may use the latest century-scale sedimentation rate as a scale for measuring the sedimentary history retrospectively. Independent evidence, such as historical records (Zhou et al., 2017), and biomarker or stratigraphic marker methodology (Hall et al., 1999; Donnelly et al., 2001; Eilers et al., 2004; Sawai, 2004) may be helpful in answering the second question. For example, Zhou et al. (2017) reconstructed a 350-year chronicle of typhoon activity at the Hainan Island based on recognition of storm depositions and a retrospective time scale derived from the sedimentation rate, which is estimated using $^{210}$Pb dating techniques and checked with historical records (Fig. 3).

### Table 1. Relationships among K, confidence level and tolerance (CNS, 1996)

<table>
<thead>
<tr>
<th>$\alpha$ (tolerance)/%</th>
<th>1–$\beta$ (confidence)/%</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>99</td>
<td>2.327</td>
</tr>
<tr>
<td>2</td>
<td>98</td>
<td>2.054</td>
</tr>
<tr>
<td>5</td>
<td>95</td>
<td>1.645</td>
</tr>
<tr>
<td>10</td>
<td>90</td>
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</tr>
<tr>
<td>20</td>
<td>80</td>
<td>0.842</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>0</td>
</tr>
</tbody>
</table>

![Fig. 2. Illustration of four cases showing the residual activities vs. the lower limit of detection (LLD, $\alpha=0.05$) of an $\alpha$ spectrometer. The grey bar is the LLD with the standard deviation of detected activities between 0.05 dpm/g and 0.40 dpm/g. Cases 1, 2, 3 and 4 represent $C_0$=10.0 dpm/g, 5.0 dpm/g, 2.5 dpm/g and 1.0 dpm/g, respectively.](image)

![Fig. 3. Retrospective time based on the sedimentation rate derived with $^{210}$Pb dating vs. the time recorded in historical literature (data from Zhou et al., 2017).](image)

### 4 The "loss of age" phenomenon in $^{210}$Pb dating

According to incomplete statistics, there are data from nearly 400 cores published since 1980 regarding the modern sedimentation rate on the coast of the Yellow Sea, East China Sea and the adjacent continental shelf (Li et al., 2012). Most of the data were based on $^{210}$Pb dating and expressed primarily as the apparent sedimentation rate (i.e., a unit of cm/a was used). We find that when dividing the length of the decay segment of the $^{210}$Pb pro-
file of the cores (i.e., above background) by the apparent sedimentation rates, the result is often less than 100 years (Fig. 4) with a mean value of only 40±20 years (Fig. 5). This finding indicates that when the $^{210}$Pb dating techniques that can nominally date the century-scale are applied to modern marine sedimentary environments, they record a sedimentary history of less than 100 years. Through analysis, we suggest the following four possible reasons.

### 4.1 Sediment compaction effect

If we directly divide the length of a decay segment of a core by the apparent sedimentation rate to calculate the age period, the implied assumption of this data processing is that the bulk density of the sediment from the top to the bottom of the core is constant. In fact, due to the compaction effect (including natural compaction and mechanical compaction during sampling), the porosity of sediments in cores gradually decreases from the surface to the bottom, and the corresponding bulk density gradually increases. From the published literature, the bulk density of the surface sediments in marine environments is primarily between 0.5 and 0.9 g/cm$^3$ (Flemming et al., 2000), whereas the bulk density at the bottom layer of cores is primarily between 1.1 and 1.3 g/cm$^3$. Therefore, using a single bulk density value for depth correction significantly increases the corrected length of cores (Zou et al., 1982). Figure 6 shows three cores collected from the East China Sea. After correction, the length of the cores increases by 1/4 to 1/2.

In fact, the sediment flux (g/(cm$^2$·a)) is the best way to represent the sedimentation rate. It can effectively avoid the calculation error caused by the compaction effect and the variation in sediment bulk density. Fan et al. (2000) used published data and gave the following empirical equations to derive the mass depth of a complete core from the water content of surface sediment under the condition of continuous deposition:

![Figure 4](https://example.com/fig4.png)

**Fig. 4.** One literature case showing that the periods derived with the apparent sedimentation rate are much less than 100 years (data from Liu et al., 2009).
The depth correction for three cores sampled in the East China Sea (data from Zou et al., 1982).

4.2 Effect of instrument measurement accuracy on the judgment of the background layer

In actual studies, researchers have primarily used Procedure II to determine the $^{210}$Pb background. From published studies, the background $^{210}$Pb$_{eq}$ in inner shelf muds of the Yellow Sea and East China Sea is approximately 1.0 dpm/g, whereas the $^{210}$Pb$_{ex}$ of surface sediments is 1–10 dpm/g. Figure 7 illustrates an example under ideal conditions. With ages of sediments increasing downward, the $^{210}$Pb$_{total}$ specific activity value decreases due to $^{210}$Pb$_{ex}$ decay. Comparing the four curves in Fig. 7, we find that the smaller the surface $^{210}$Pb$_{eq}$ is, the faster it approaches $^{210}$Pb$_{eq}$ and the difference between $^{210}$Pb$_{eq}$ and $^{210}$Pb$_{ex}$ also becomes more difficult to distinguish. Figure 8 is another illustration similar to Fig. 7. We randomized the errors of the $^{210}$Pb$_{total}$ measurements of different ages (–5% to +5%). As shown, for cores with a small specific activity, after two to three half-lives, analytical instruments can scarcely distinguish the difference between the $^{210}$Pb$_{eq}$ and the $^{210}$Pb$_{ex}$. The estimated age of the $^{210}$Pb$_{eq}$ layer may decrease by a large amount. For example, when the surface $^{210}$Pb$_{ex}$ is 1.0 dpm/g and 2.5 dpm/g, the corresponding age of the assigned $^{210}$Pb$_{eq}$ is only 60 years and 80 years, respectively (Fig. 8).

In fact, the examples shown in Fig. 7 are ideal cases. Limited by the analytical capabilities of laboratory instruments and by research funds, many early $^{210}$Pb dating data were unevenly measured and scarcely distributed along the depths of cores. If the measurement interval for $^{210}$Pb activity at the lower parts of cores is greater than 10 cm, the error in judging the background layer is more difficult to distinguish. Figure 8 is another illustration similar to Fig. 7. We randomized the errors of the $^{210}$Pb$_{total}$ measurements of different ages (–5% to +5%). As shown, for cores with a small specific activity, after two to three half-lives, analytical instruments can scarcely distinguish the difference between the $^{210}$Pb$_{eq}$ and the $^{210}$Pb$_{ex}$. The estimated age of the $^{210}$Pb$_{eq}$ layer may decrease by a large amount. For example, when the surface $^{210}$Pb$_{ex}$ is 1.0 dpm/g and 2.5 dpm/g, the corresponding age of the assigned $^{210}$Pb$_{eq}$ is only 60 years and 80 years, respectively (Fig. 8).

In fact, the examples shown in Fig. 7 are ideal cases. Limited by the analytical capabilities of laboratory instruments and by research funds, many early $^{210}$Pb dating data were unevenly measured and scarcely distributed along the depths of cores. If the measurement interval for $^{210}$Pb activity at the lower parts of cores is greater than 10 cm, the error in judging the background layer is larger. Therefore, we suggest that under the present circumstances in which laboratory analytical capabilities and research funds have increased significantly, the measurement interval of $^{210}$Pb activity should be reduced as much as possible, and it is best to use uniform intervals.

4.3 Selection of the $^{210}$Pb background value and its effect on dating results

We find that a small difference in the $^{210}$Pb background can cause a significant discrepancy in the calculated sedimentation
rate and the recorded age limit of the $^{210}$Pb decay segment. Figure 9 shows a core taken in the East China Sea, with a typical two-segment vertical distribution of $^{210}$Pb specific activity. The top slope segment reflects the decay process of $^{210}$Pb$_{ex}$. The bottom straight segment reflects the condition of $^{210}$Pb$_{eq}$. The backgrounds obtained by two procedures mentioned in Section 3.1 are 1.1 dpm/g and 1.2 dpm/g, respectively. Although their difference is less than 10%, the calculated age periods of the slope segment using three combinations of backgrounds and dating models differ by 20 years. CRS model with larger value of background activities will give elder dating results than with smaller background activities, and dating results derived with CRS model are generally older than with CIC model.

To reduce the effect of the $^{210}$Pb background value on the dating result as much as possible, we suggest measuring the $^{226}$Ra and $^{210}$Pb activity at the same time, which can effectively avoid the uncertainty error brought by the above empirical selection.

4.4 Disturbance, erosion and migration effects

Taking the Zhe-Min inner shelf mud zones as examples, the sediments there primarily originate from a portion of Changjiang River sediments transferred to the sea that are transported southward by the Zhe-Min coastal current (Qin et al., 1996; Liu et al., 2006; Gao and Collins, 2014). Summer typhoons, winter waves and strong winds occur frequently, which can disturb sediments and produce erosional transportation (Dai, 1992; Xie et al., 2001). In other words, the behavior of $^{210}$Pb in the Zhe-Min inner shelf mud zones can hardly satisfy the condition required by the $^{210}$Pb dating model (Appleby and Oldfield, 1983; Wan, 1997). Once there is an extreme weather event, sedimentary records are lost and reflect a “loss of age” of $^{210}$Pb records in cores.

5 Discussion

5.1 Suitability of the $^{210}$Pb dating techniques in marine environments

With the CIC model or the CRS model, the use of the $^{210}$Pb dating techniques must follow several basic assumptions (Wan, 1997). (1) Sediments must be a closed system, and their source,
According to this assumption, we can infer that the total accumulative $^{210}\text{Pb}_{ex}$ in sediments should be $12.66-66.11$ dpm/cm$^2$ (i.e., the product of the atmospheric deposition flux and the average lifetime of $^{210}\text{Pb}$; 1 Bq=60 dpm), knowing that the global atmospheric $^{210}\text{Pb}_{ex}$ deposition flux is 0.18–0.94 Bq/(m$^2$·d) (Liu, 2010). Oldfield et al. (1978) found substantial evidence from two cores taken in a lake in that although their sedimentation rate differed by three times, the total accumulative $^{210}\text{Pb}_{ex}$ was essentially the same.

From the analysis in Sections 5.1 and 5.2, it is difficult for marine sediments to satisfy the requirements of the CRS model. However, this situation may bring new information to marine sedimentary studies. Using the core shown in Fig. 9 as an example, the two-segment $^{210}\text{Pb}$ profile lacks an upper mixing layer. In the past, this phenomenon was often interpreted as a weak mixing and weak disturbance in the water-sediment interface. However, if the core once had a mixing layer but was eroded, it would also form a two-segment $^{210}\text{Pb}$ profile. How do we determine which explanation is closer to reality? We analyzed the rela-

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**Fig. 9.** The $^{210}\text{Pb}$ specific activities profile of a short core collected in the East China Sea (a) and dating results of three combinations of background values and dating models (b). Left panel: the red label indicates the value of $^{210}\text{Pb}_{ex}$ determined with Procedure I, whereas the black label indicates the value of $^{210}\text{Pb}_{ex}$ determined with Procedure II (see the text in Section 3.1 for explanation). Right panel: blue cross, black triangle and red cycle represent combinations of CIC model ($^{210}\text{Pb}_{eq}$=1.1 dpm/g), CRS model ($^{210}\text{Pb}_{eq}$=1.1 dpm/g) and CRS model ($^{210}\text{Pb}_{eq}$=1.2 dpm/g), respectively. The corresponding dating results for the layer of background are 64 years, 74 years and 85 years.
tion between $A_A$ (the total accumulative $^{210}\text{Pb}$ in sediments) and the atmospheric $^{210}\text{Pb}$ deposition flux in the same period; we found that they are essentially equal. Thus, we could infer that the sedimentary environment of this core was stable and lacked any post-deposition disturbance, resuspension and erosion.

6 Conclusions

(1) Direct $^{210}\text{Pb}$ dating works best for a time scale with an upper limit of 110–155 years. The "prolonging" application is acceptable only if the sedimentary environment in the past several hundred years was stable and the sedimentation rate was generally constant, and verification with independent evidence (such as historical records or biomarker methodology) is needed.

(2) Due to the compaction effect that occurs during deposition and sampling, the widely used "apparent sedimentation rate" (cm/a) will cause inherent and systematic errors in $^{210}\text{Pb}$ dating. We recommend the sediment mass flux (g/(cm$^2$-a)) instead of the apparent sedimentation rate (cm/a) for $^{210}\text{Pb}$ dating.

(3) In addition to the compaction effect, the uncertainty of activity measurement and the post-deposition erosion and migration can also lead to "loss of age" in $^{210}\text{Pb}$ dating. Reducing the interval of sub-samples for $^{210}\text{Pb}$ activity measurement and measuring the $^{226}\text{Ra}$ activity concurrently can effectively lessen the $^{210}\text{Pb}$ dating error.

(4) Open marine environments cannot strictly satisfy the requirements for the $^{210}\text{Pb}$ dating model. However, from the aspect of marine environment characteristics, $^{210}\text{Pb}$ dating data may provide useful information on issues such as the material sources of marine sediments and the post-deposition erosion and disturbance.

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Estimation of genetic parameters for upper thermal tolerance and growth-related traits in turbot Scophthalmus maximus using the Bayesian method based on Gibbs sampling

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Abstract

In order to carry out the genetic improvement of turbot upper thermal tolerance, it is necessary to estimate the genetic parameters of UTT (upper thermal tolerance) and growth-related traits. The objective of this study was to estimate genetic parameters for BW (body weight) and UTT in a two-generational turbot (Scophthalmus maximus L.) pedigree derived from four imported turbot stocks (England, France, Denmark and Norway). A total of 42 families including 20 families from G3 generation and 22 families from G4 generation were used to test upper thermal tolerance (40–50 animals per family) in this study and the body weight of individuals were measured. The heritability of BW and UTT and the correlation between these two traits were estimated based on an individual animal model using Bayesian method based on two types of animal models with and without maternal effects. These results showed that the heritabilities for BW and UTT and phenotypic and genetic correlations between the two traits estimated from model without maternal effects were 0.239±0.141, 0.111±0.080, 0.075±0.026 and -0.019±0.011, respectively. The corresponding values from model with maternal effects were 0.203±0.115, 0.055±0.026, 0.047±0.034 and -0.024±0.028, respectively. The maternal effects of BW and UTT were 0.050±0.017 and 0.013±0.004, respectively. The maternal effects had a certain influence on the genetic evaluation of the two traits. The findings of this paper provided the necessary background to determine the best selection strategy to be adopted in the genetic improvement program.

Key words: heritability, genetic correlation, thermotolerance, body weight, Turbot, Bayesian inference


1 Introduction

Turbot (Scophthalmus maximus L.) is an important commercial flatfish, which is widely distributed in the Baltic, Black, and Mediterranean Seas (Zhang et al., 2014; Wang et al., 2015; Wang and Ma, 2016). Although turbot industry in China has made great progress in recent years, there are still some problems to be solved about further promoting the industrialization development, for example, temperature tolerance. Turbot is a type of cold-water fish with strict requirements for environmental temperature. Therefore, it is particularly susceptible to thermal stress (Zhang et al., 2014). Suitable growth temperature for different size turbot is between 16°C and 24°C (Xu et al., 2015). In general, the tolerance to high temperatures for adult turbot, under ideal environmental conditions, for example, good air and flowing water, can reach 25–26°C but the temperatures can not be maintained for a long time. The high and low water temperatures on growth are 21–22°C and 7–8°C, respectively (Ma et al., 2012). Temperature, as the abiotic master factor for fishes, affects almost all biochemical, physiological and life history activities of fishes (Beitinger et al., 2000). The unsuitable high temperatures result in stress response against fish and cause low disease resistance, growth rate and reproductive activity (Dominguez et al., 2004). In culture areas of turbot in North China, the natural sea water temperatures usually exceed 26°C during the whole summer (May to September) (Zhang et al., 2014). Obviously, the temperatures are unsuitable for rearing turbot (optimal growth temperatures around 16°C). In order to solve the problem, the current cooling method was mainly used with seawater pumped...
from deep wells. This method was effective, but its shortcomings were also obvious. The extraction of groundwater is not only a waste of energy but also causes great environmental pressures; the groundwater exceeding extraction can cause a large-scale decrease in the water table and subsequent water shortage. Thus, the genetic improvement of turbot upper thermal tolerance is necessary to sustain the further development of the industry.

Estimates of genetic parameters for selected traits are an important and fundamental work in fish breeding programs (Fu et al., 2015; Sun et al., 2015). As one of the most important genetic parameter, heritability is a key quantitative indicator in quantitative genetics, which is applied to the study of genetic mechanism of selected traits based on the phenotype data measuring. In addition, it also plays an extremely vital role in estimating breeding value, formulating selection index, predicting selection response, comparing breeding methods and determining reasonable breeding plans (Sun et al., 2015). Genetic correlation, in quantitative genetics, is another important basic genetic parameter, which is used to explore the correlations between different traits resulting and various genetic causes. It plays an important role in determining the genetic basis for indirect selection, predicting indirect selection response, comparing selection effects in different environments and formulating integrated selection index (Sun et al., 2015). The usual methods for estimating genetic parameters include maximum likelihood (ML) (Hartley and Rao, 1967), restricted maximum likelihood (REML) (Patterson and Thompson, 1971), average information restricted maximum likelihood (AI-REML) (Jensen et al., 1997), minimum variance quadratic unbiased estimation (MIVQUE) (Swallow and Searle, 1978), minimum norm quadratic unbiased estimation (MINQUE) (Rao, 1971) and Bayesian methods (Harville, 1974; Gianola and Fernando, 1986). Each has its advantages and disadvantages. Among them, maximum likelihood (ML), restricted maximum likelihood (REML) and Bayesian methods are often preferred over other methods for estimating variance components in animal breeding (Hoeschele, 1989). In recent years, with increasing attention being paid to genetic improvement in flatfish, some genetic parameters (mainly heritability and genetic correlation) for selected traits were reported for a variety of flatfish (Blonk et al., 2010; Wang et al., 2010; Liu et al., 2011a, b, 2015, 2016a, b, c; Tian et al., 2011; Zhang et al., 2014; Xu et al., 2015; Guan et al., 2016). The documents showed that these reported genetic parameters were estimated by restricted maximum-likelihood method (REML) (Blonk et al., 2010; Wang et al., 2010; Liu et al., 2011a, b, 2016a, b, c; Tian et al., 2011; Zhang et al., 2014; Xu et al., 2015; Guan et al., 2016), average information restricted maximum likelihood (AI-REML) (Liu et al., 2015) and minimum norm quadratic unbiased estimation (MINQUE) (Tian et al., 2011). However, the estimation of genetic parameters using Bayesian methods has not been reported in flatfish. Bayesian methods using Gibbs sampling (GS) and a Monte Carlo numerical integration technique by simulation are an important evaluation method of genetic parameters in the field of animal breeding (Wang et al., 2011). Gibbs sampling is a method of numerical integration that allows inferences to be made about joint or marginal densities, even when those densities cannot be evaluated directly (Wang et al., 2011). Compared with other evaluation methods, Bayesian method has a huge advantage in genetic parameters estimation, particularly when data do not satisfy normal distribution (Wang et al., 2011). Usually, thermotolerance can be regarded as a kind of threshold character. The threshold character data are difficult to meet normal distribution. Therefore, it is highly suitable to estimate the genetic parameters of thermotolerance character using Bayesian method.

The ultimate goal of genetic improvement, regardless of any selected traits, is to obtain greater economic benefits. Obviously, it is not important for thermotolerance selection without considering growth rate. Thus, turbot selective breeding program for thermotolerance and fast-growth was conducted at the Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences in 2007. In the present study, we evaluated the heritabilities of turbot body weight (BW) and upper thermal tolerance (UTT) as well as the genetic correlations among them by full- and half-sib family analysis using Bayesian method via Gibbs sampling. The main objective of this paper is to provide some fundamental insight for further selection through the genetic parameters of the two traits.

2 Materials and methods

2.1 Genetic material and production of family

The data were derived from a breeding program initiated in 2007 by the Yellow Sea Fisheries Research Institute, Qingdao, China. Four imported turbot stocks from England, France, Denmark and Norway from June 2002 to September 2003 were used to establish base populations for selective breeding by genetic background analysis of the four stocks. A total of 170 brood stocks including 68 males and 102 females were obtained. These brood stocks were individually tagged with the passive integrated transponders (PIT) in December 2006 (G1 generation). In April 2007, 56 F1 full-sib families were produced using a nested mating design with one male and two females by Yantai Tianyuan Aquatic Limited Corporation, Yantai, China. At 12 months of age, 20 randomly selected families from the F1 families were used to test upper thermal tolerance (40–50 animals per family), and then the upper thermal tolerance (UTT) for each individual fish was calculated as cumulative thermal exposure in degree hours (deg hr) as followed Perry et al. (2005). An F2 breeding program was developed based on UTT of the F1 animals and pedigree relationship, and 42 full-sib families were obtained in April 2010. Follow the same procedure as F1 thermal tolerance test, 22 randomly selected families from the F2 families were selected to test upper thermal tolerance (40–50 animals per family) when the F2 families were reared up to 12 months of age. The two-generation families were reared in separate tanks until tagging. When the fish were reared up to 3 months of age, samples of 250–300 fish were randomly selected from each tank for tagging using the visible implant elastomer (VIE) (for distinguishing different families) and then stocked communally. They were individually tagged with the passive integrated transponders (PIT) again (for distinguishing families and individuals) when all tagged fish with VIE were communally reared up to 9 months of age. To obtain similar rearing conditions for all F2 and F3 families at different breeding stages, some effective measures were taken to standardize both the stocking density of fish and the environment. The standard operating procedures were same as Wang et al. (2010).

2.2 Experimental procedure

A total of 42 families including 20 families from G1 generation and 22 families from G2 generation were used to test upper thermal tolerance (40–50 animals per family) in April 2008 and April 2011, respectively. Fish from each family used in thermal tolerance challenges were reared in separate tanks for 1 week at 15°C before exposure to an acute thermal challenge. During the entire experimental period, the temperature of fish subjected to chronic thermal shock was increased by 13°C (1°C per 12 h up to
26°C and 1°C per 24 h between 26°C and 28°C), then held at 28°C until the end of the experiment (the lethal temperature for the turbol was 28°C). The experiment was finished when two-thirds of all individuals have lost their activity. The time to loss of activity (LOA) was determined based on the reaction of fish when the water is agitated for 10 s. In the experimental periods, the still water, aerated cultures and automatic thermostat control were used to culture the tested fish. The fish activities were observed every hour and the individuals of loss of activity were timely removed. In the thermal challenge, some variability of the temperature of the time to loss of activity was observed. In order to correct for these differences, the upper thermal tolerance (UTT) was calculated as cumulative thermal exposure in degree hours (deg hr) as

\[ \text{UTT} = \sum_j (T_j - T_a), \]

where \( j \) represents each hour up to time to loss of activity (LOA) for each individual fish, \( T_j \) is the experimental temperature at each hour and \( T_a \) is the acclimation temperature (15°C). This method has been reported previously (Perry et al., 2005; Zhang et al., 2014). The body weight (BW, g) was also recorded for each individual fish at the beginning of the experiment. All the families shared the same environment and handling during the entire experimental period in the high temperature conditions to ensure that the eventual differences among them were detected.

### 2.3 Statistical and genetic analysis

The Kolmogorov-Smirnov test (K-S test) was used to test the normality of data for UTT and BW from each family before the data are analyzed. The Kolmogorov-Smirnov Z values and two-tailed probability (P) values of each family were calculated by using SPSS 13.0 software package (Norusis, 2009). The family data are considered to be normal distribution when P>0.05 and non-normal distribution when P<0.05.

Variance components, heritabilities, and genetic correlations with standard errors for UTT and BW traits were estimated using Gibbs sampling (GS) in Multiple Trait Gibbs Sampling in Animal Models (MTGSAM) programs (Harville, 1974; Gianola and Fernando, 1986) using the two types of animal models. Maternal genetic effect was included in Model 2 and was not included in Model 1. The two models can be written as:

**Model 1:** \( y_{ijk} = u + a_i + f_j + g_k + e_{ijk} \)

**Model 2:** \( y_{ijkl} = u + a_i + f_j + g_k + m_l + e_{ijkl} \)

where \( u \) is the population mean, \( y_{ijk} \) is the measured values of UTT and BW, \( a_i \) is the additive genetic effect of individual as the random effect, \( f_j \) is full-sib family random effect, \( g_k \) is generation effect \((k=1 \text{ or } 2)\), \( m_l \) is maternal genetic effect, \( e_{ijk} \) and \( e_{ijkl} \) are the random residual. In matrix notation the model can be written:

\[ y = Xb + Zu + e. \]

where \( y \) is the vector of observations of each trait, \( b \) is the vector of fixed effects, \( u \) is the vector of random effects, \( X \) and \( Z \) are known design matrices assigning the observations to levels of \( b \) and \( u \) respectively. The mathematical expectation and variance was defined as

\[ E (u) = 0, \quad E (e) = 0, \quad E (y) = Xb. \]

\[ \text{Var} \left( \begin{array}{c} u \\ e \end{array} \right) = \begin{pmatrix} G_a \otimes A & 0 \\ 0 & R_e \otimes I \end{pmatrix}, \]

\[ G_0 = \begin{pmatrix} r_{11} & r_{12} \\ r_{21} & r_{22} \end{pmatrix}, \]

where \( G_a \) is the genetic variances-covariances matrix of UTT and BW, \( R_e \) is the residual variance-covariance matrix of the two traits, \( \otimes \) is Kronecker product, \( \text{Var} \ (y) = X^\prime A X + I \sigma_e^2 . \)

**A** is the additive genetic relationship matrix, **I** is an identity matrix, and \( \sigma_a^2 \) is additive genetic variance.

The equations of two-trait animal model are

\[ \begin{bmatrix} X^\prime R^{-1} X & X^\prime R^{-1} Z \\ Z^\prime R^{-1} X & Z^\prime R^{-1} Z + A - 1 \otimes G^{-1} \end{bmatrix} \begin{bmatrix} \hat{b} \\ \hat{a} \end{bmatrix} = \begin{bmatrix} X^\prime R^{-1} y \\ Z^\prime R^{-1} y \end{bmatrix}, \]

\[ X = \begin{bmatrix} X_1 \\ 0 \\ X_2 \end{bmatrix}, \quad Z = \begin{bmatrix} Z_1 \\ 0 \\ Z_2 \end{bmatrix}, \quad b = \begin{bmatrix} \hat{b}_1 \\ \hat{b}_2 \end{bmatrix}, \quad a = \begin{bmatrix} \hat{a}_1 \\ \hat{a}_2 \end{bmatrix} \quad \text{and} \]

\[ \hat{y} = \begin{bmatrix} \hat{y}_1 \\ \hat{y}_2 \end{bmatrix}. \]

Heritability \((h^2)\) and genetic correlation \((r_g)\) can be written:

\[ h^2 = \frac{\sigma_a^2}{\sigma_p^2}, \quad r_g = \frac{\text{cov}(a_1, a_2)}{\sqrt{\sigma_a^2 \cdot \sigma_a^2}}, \]

where \( \sigma_a^2 \) is additive genetic variance, \( \sigma_p^2 \) is phenotype variance, \( a_1 \) and \( a_2 \) are additive genetic effects of \( x \)- and \( y \)-traits and \( \text{cov}(a_1, a_2) \) is covariance of \( a_1 \) and \( a_2 \).

Bayesian inference facilitates obtaining the marginal posterior or probability density of the genetic parameters, and from these densities, the calculation of the errors in the parameter estimates gets more information. In this study, a total Gibbs chain length of 5 000 samples for each analysis was defined with a burn-in period of 200 and a thinning interval of 30 uninformative (flat) priors were used for additive genetic and residual (co)variances. The phenotypic correlations between UTT and BW were estimated as Pearson’s correlations by using SPSS 13.0 software package (Norusis, 2009).

### 3 Results

#### 3.1 The descriptive statistics of traits

The descriptive statistics for UTT and BW were presented in Table 1. The values of UTT ranged from 962.000°C to 2 292.000°C and that of BW from 51.000 g to 153.000 g. The coefficient of variation (CV) of the two traits were high, greater than 15%, the trait BW even up to 20.370%, which indicated that there existed some very significant differences between individual in the two traits and had greater potential for genetic improvement.

#### 3.2 The normal distribution test for data

The normal distribution test of the two variables was done with Kolmogorov-Smirnov test. Details of the parameters may be observed in Table 2. The Kolmogorov-Smirnov Z values for BW ranged from 0.477 to 0.819 and that for UTT from 1.330 to 1.559. All Asymp.Sig. (2-tailed) values \((P \text{ value})\) for BW ranged from 0.616 to 0.973 and were higher than 0.05. On the contrary, most Asymp.Sig. (2-tailed) values for UTT were lower than 0.050 except for Family 22 (0.053), Family 3 (0.053), Family 5 (0.056) and Family 4 (0.058) from G generation (the other \( P \) values ranged from 0.016 to 0.050).
Estimation of genetic parameters

Estimates of heritability for BW and UTT and the phenotypic and genetic correlations between the two traits estimated from two types of animal models were given in Table 3. The estimate of heritability for UTT, estimated from Model 1 that did not include maternal genetic effect, was low (0.111±0.080) and was no significant difference from zero \((P>0.05)\), whereas that for BW was moderate (0.239±0.141) and was significant difference from zero \((P<0.05)\). The phenotypic and genetic correlation between UTT and BW estimated from Model 1 was a low positive value (0.075±0.026) and a weak negative value (–0.019±0.011), respectively. The estimate of heritability for UTT, estimated from Model 2 that included maternal genetic effect, was lower (0.055±0.026) than that from Model 1 (0.111±0.080) and maternal genetic effect was 0.013±0.004, whereas that for BW was 0.203±0.115 and maternal effect was 0.050±0.017. The phenotypic and genetic correla-
Bayesian analysis method has a huge advantage in heritability computation including probability statements. Because of the inferences obtained by employing the posterior and greater flexibility than the usual likelihood estimates, mainly animal breeding, restricted maximum likelihood (REML) models in recent years. Bayesian analysis via Gibbs sampling is an appropriate alternative for estimating (co)variance components and genetic parameters. It can provide reasonable estimates and greater flexibility than the usual likelihood estimates, mainly because of the inferences obtained by employing the posterior marginal distributions. Any features of this distribution can be computed including probability statements. Obviously, Bayesian analysis method has a huge advantage in heritability estimation, particularly when data do not fit a normal distribution. Based on the data characteristics in this study, it is more reasonable to select Bayesian analysis for more accurate genetic evaluation.

In this study, a total of 1 260 individuals from 42 families (two generations) were used to estimate genetic parameters for UTT and BW using Bayesian method based on two types of animal models with and without maternal effects. Heritabilities for BW and UTT and phenotypic and genetic correlations between the two traits estimated from model without maternal effects (Model 1) were 0.239±0.141, 0.111±0.008, 0.075±0.026 and -0.019±0.011, respectively. The corresponding values from model with maternal effects (Model 2) were 0.203±0.115, 0.055±0.026, 0.047±0.034 and -0.024±0.028, respectively. Obviously, the maternal effects had a certain influence on the genetic evaluation of the two traits. The existence of maternal effects should be the reason for separate family rearing before tagging. This speculation was consistent with the conclusion of some studies that maternal effects were detected when families were reared separately until tagging size (Nielsen et al., 2010; Ninh et al., 2011; Dong et al., 2015). In addition, other studies reported that the maternal effects were negligible when families were reared communally from newly hatched larvae (Vandeputte et al., 2004; Ninh et al., 2011), which further confirmed that maternal effects could be eliminated when fish were reared in communal stocks. Therefore, it is necessary, for genetic evaluation of breeding traits, to take into account maternal effects in analysis model when all families were not reared communally from newly hatch.

The economic importance of upper thermal tolerance in aquaculture species has given rise to some experiments in the last few decades with the aim of genetic improvement (Perry et al., 2005; Liu et al., 2011b; Zhang et al., 2014). Perry et al. (2005) estimated genetic (co)variance parameters for body weight (BW) and upper thermal tolerance (UTT) in a three-generational rainbow trout (Oncorhynchus mykiss) pedigree derived from two commercial strains using restricted maximum likelihood (REML). The heritability for BW and UTT is 0.460±0.040 and 0.410±0.070, respectively. The genetic correlation between two traits is -0.30±0.080 and the phenotypic correlation is 0.060±0.013 (Perry et al., 2005). Liu et al. (2011b) used 753 animals from 40 families (G2 generation) of turbot to estimate the genetic parameters of juvenile BW (mean value: (7.300±3.600) g) and UTT with average information restricted maximum likelihood method (AI-REML). The heritability of BW and UTT is 0.220±0.090 and 0.026±0.034, respectively. The standard error for the estimated heritability of upper thermal tolerance (0.034) is larger than the estimated heritability (0.026). The phenotypic and genetic correlation between the two traits is 0.040 and -1.000, respectively (Liu et al., 2011b).

Table 3. Heritability for the growth and upper thermal tolerance traits and phenotypic ($r_p$) and genetic ($r_g$) correlations between the two traits estimated from two animal models

<table>
<thead>
<tr>
<th>Model</th>
<th>Trait</th>
<th>Heritability ($h^2$)</th>
<th>Maternal effects</th>
<th>$r_p$</th>
<th>$r_g$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1</td>
<td>UTT</td>
<td>0.111±0.080</td>
<td>-0.019±0.011</td>
<td>0.075±0.026</td>
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<tr>
<td></td>
<td>BW</td>
<td>0.239±0.141</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Model 2</td>
<td>UTT</td>
<td>0.055±0.026</td>
<td>0.013±0.004</td>
<td>-0.024±0.028</td>
<td>0.047±0.034</td>
</tr>
<tr>
<td></td>
<td>BW</td>
<td>0.203±0.115</td>
<td>0.050±0.017</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In a successful breeding program, genetic parameters of the selected traits should be correctly estimated so that suitable breeding programmes could be planned (Narinc et al., 2010). The accuracy estimations of genetic parameters depend on the use of a correct model, a large sample size and reliable statistical methods (Wang et al., 2011). During the experiment (12 months of age), the sex of turbot was unable to be distinguished and the difference between female and male turbot in growth was not significant (P>0.05) (Wang et al., 2014), so the effect was not taken into account in the model. The heritabilities of traits were generally estimated using two types of models with and without maternal genetic effects. The documents showed that the maternal effects in some studies, for example, estimation of genetic parameters for growth-related traits in common carp (Vandeputte et al., 2004; Ninh et al., 2011), were negligible; on the contrary, there were statistically significant maternal effects in other studies, for example, genetic parameters for growth and survival in common carp (Nielsen et al., 2010; Ninh et al., 2011; Dong et al., 2015). In the present study, two types of animal models with excluding and including maternal genetic effects (Model 1 and Model 2) (the two models all include additive genetic effect, full-sib family effect and generation effect) were used to estimate genetic parameters. Appropriate family numbers and large sample sizes are generally required to estimate heritabilities and genetic correlations accurately. Simulation studies revealed that the estimation value was more accurate for larger sample size (Wang et al., 2011). In this paper, a total of 1 260 individuals from 42 families (two generations) were used to estimate genetic parameters for UTT and BW. The sample size was moderate for genetic evaluation due to the limitation of experimental conditions. There are two main reasons for the limitation: firstly, for the sake of ensuring common conditions in the entire experiment and secondly the consumption of expensive experimental materials. In addition, the body weight data from each family fit the normal distribution based on Kolmogorov-Smirnov test but the UTT data mostly do not (only three sets of UTT data were accorded with normal distribution). Based on the data characteristics in this study (sample size and normality), it is very important to choose reasonable approach for accurate genetic evaluation. In the context of animal breeding, restricted maximum likelihood (REML) and Bayesian method have been found preferable over others for estimating variance and covariance components in mixed linear models in recent years. Bayesian analysis via Gibbs sampling is an appropriate alternative for estimating (co)variance components and genetic parameters. It can provide reasonable estimates and greater flexibility than the usual likelihood estimates, mainly because of the inferences obtained by employing the posterior marginal distributions (Faria et al., 2007). Any features of this distribution can be computed including probability statements. Obviously, Bayesian analysis method has a huge advantage in heritability estimation, particularly when data do not fit a normal distribution. Based on the data characteristics in this study, it is more reasonable to select Bayesian analysis for more accurate genetic evaluation.
traits is 0.093±0.029 and the genetic correlation is ~0.044±0.239. From these results, it can be seen that the differences of four parameters (including two heritabilities, phenotypic correlation and genetic correlation) between current research (the conclusions from models including maternal effects) and rainbow trout (*Oncorhynchus mykiss*) are greater than the differences between current research and turbot (*Scophthalmus maximus* L.). We speculated that this discrepancy is mainly due to the differences between species. Compared with two kinds of conclusions from turbot, the estimated heritability of UTT, in the present study, is higher than the estimates of both Liu et al. (2011b) and Zhang et al. (2014), and the heritability of BW is less than the estimates of both Liu et al. (2011b) and Zhang et al. (2014). The phenotypic correlation between UTT and BW is situated between Liu et al. (2011b) and Zhang et al. (2014) estimates and the genetic correlation is higher than the estimates of both Liu et al. (2011b) and Zhang et al. (2014). It is speculated that this kind of intraspecific differences is mainly attributed to the number of individuals contributing to the estimates, the use of different statistical methods and models, different source populations and different developmental periods of samples used. Among these factors, special attention should be paid to the difference of developmental periods of samples used. Liu et al. (2011b) and Zhang et al. (2014) have studied upper thermal tolerance of juvenile turbot, and this paper has explored upper thermal tolerance of adult turbot.

Estimated heritability could be classified as low (0.05–0.15), medium (0.20–0.40), high (0.45–0.60) and very high (>0.65) levels (Cardellino and Rovira, 1987; Xu et al., 2015). This study determined that the heritability of BW and UTT were 0.203±0.115 and 0.055±0.026, respectively. According to this grading standard, the heritability of BW and UTT could be defined as medium and low heritability, respectively. Moderate heritability on the BW trait demonstrated promising effects on genetic improvement in select breeding programs of the turbot industry. Clearly, the selection methods are very flexible for genetic improvement of BW. For the UTT traits with low heritability, the determination of the selection strategy requires careful consideration, and family selection is to be preferred, provided that common environmental effects are kept at a low level (Rye et al., 1990; Wang et al., 2010). The magnitude of correlations was categorized as low (0.0–0.40), medium (0.45–0.55) and high (0.60–1), independent of the sign (Cardellino and Rovira, 1987; Xu et al., 2015). In this study, the genetic and phenotypic correlations between BL (body length) and BW were −0.024±0.028 and 0.047±0.034, respectively. Obviously, it was positive and low in magnitude for the phenotypic correlation but negative and low for the genetic correlation. It is very important to obtain the phenotypic and genetic correlations among breeding traits for the design of breeding programs (Zhang et al., 2014). Negative genetic correlation between traits might cause offsets in genetic gain from selection on single characters by economic losses in correlated traits. Additionally, the multi-trait selection breeding also has difficulty obtaining good breeding results due to offsets between the two traits. Clearly, the BW and UTT traits cannot be improved simultaneously in a selection programme. Given the negative genetic correlations between the two traits, methods of achieving simultaneous improvement of both traits require further investigation. A breeding method by first cultivating two new strains and then hybridization between the two strains may be a strategy worthy of consideration. From a breeding viewpoint, the estimated genetic parameters provided the necessary background to determine the best selection strategy to be adopted in the genetic improvement program in order to allow the selection response and efficient advancement predicted by this study.

References
MA Aijun, Huang Zhilui, Wang Xian, et al. 2012. The selective breeding of thermal tolerance family and appraisal of performance in...


Genetic parameters and response to selection for body weight in turbot (*Scophthalmus maximus*, Linnaeus)

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Abstract

Genetic parameters and response to selection were estimated for harvest body weight in turbot. The data consisted of 10,952 individuals of 508 full-sib families from three generations (G0, G1, and G2). The heritability estimates for G0, G1, and G2 were 0.11±0.08, 0.18±0.09, and 0.17±0.07, respectively. Over three generations, the heritability estimate was 0.19±0.04. Maternal and common environmental effects were 0.10±0.04, 0.14±0.04, and 0.13±0.03 within each generation and 0.12±0.01 across generations. The selection differential in growth was 18.24 g in G0 and 21.19 g in G1 corresponding to an average of 19.72 g per generation. The genetic gains were also calculated, they were 22.96 g in G1 and 11.93 g in G2, corresponding to 6.36% and 3.52% body weight. The total genetic gain after two generations was 10.10% body weight, which indicated that the selective breeding program for the body weight trait in turbot was successful.

Key words: turbot, genetic parameter, selective breeding, body weight, *Scophthalmus maximus*


1 Introduction

Turbot (*Scophthalmus maximus*, Linnaeus, 1758) is a marine fish distributed on the Atlantic coasts of Europe, including the Baltic Sea, Black Sea, and Mediterranean Sea (*Blanquer et al., 1992; Lei and Liu, 1995*). With the advantages of fast growth, high cold resistance, rich taste, and good nutrition, turbot is the most widely cultured commercial flatfish globally. Since its introduction into China from Britain as juvenile by the Yellow Sea Fisheries Research Institute in 1992, turbot aquaculture has developed into an important industry with an annual production of 56,300 t in 2013 (*National Technology Research and Development Center of Flatfish Culture Industry, 2014*). To promote the development of the turbot aquaculture industry and increase turbot production, there is a major effort in genetic improvement programs aimed at body weight traits to breed fast-growing varieties, because the commercial value of a turbot depends primarily on its body weight.

Selective breeding is a basic approach for genetic improvement that can offer the opportunity of continuous genetic gain; any gain in a core breeding group can be multiplied and expressed in millions of progeny (*Lind et al., 2012*). Selective breeding has been conducted for many aquatic species to improve the body weight trait (*O’Flynn et al., 1999; Bentsen et al., 2012; Luan et al., 2012; Sui et al., 2015*). In China, turbot breeding began more than a decade ago and two cross varieties, Danfa Turbot (Registration No. GS-02-001-2010) and Duobao No.1 (Registration No. GS-02-001-2014), have been ratified by the National Certification Committee for Aquatic Varieties of China. However, there have been no reports, to date, on genetic gains for any traits in either turbot or the newly selected turbot varieties.

A selective breeding program based on best linear unbiased prediction (BLUP) to improve harvest body weight for cultured turbot carried out by the Yellow Sea Fisheries Research Institute was initiated in 2006. The selective breeding program for turbot has now been underway for over ten years and phenotypic data for harvest body weight has been obtained for two selective generations (G1 and G2). Here, the heritability for harvest body weight was estimated based on within and across generation datasets using the restricted maximum likelihood (REML) method; the response to selection after two generation selection was calculated in the form of selection differential and genetic gains. The results provide a reference point with directive significance for further turbot selection breeding work.

2 Materials and methods

2.1 The environment

The breeding program was performed at the Genetic Breed-
ing Center of Seawater Flatfish (36°40′24″N, 121°09′00″E), Yellow Sea Fisheries Research Institute, Haiyang City, Shandong Province, China. The progenies were cultured in two farms: one in the Genetic Breeding Center of Seawater Flatfish and the other one (36°54′14.10″N, 121°48′37″E) in Rushan City, Shandong Province, China.

2.2 Production of base generation (G0)

The base turbot generation (G0) was bred by introduced populations from five countries (Denmark, France, Spain, Britain, and Chile), all of which were introduced as about 5 cm long juvenile in 2003 and 2004. Healthy individuals that fed well and exhibited normal morphology were chosen as the brood stock. The brood stock were reared in 5 m×5 m×0.6 m (L×W×H) ponds at a density of 2–3 kg/m². Three months before breeding, illumination and water temperature in the culture ponds were controlled to stimulate gonad development. Illumination intensity on the water was 200–600 lx and the time increased gradually from 6 h to 10 h per day. Water temperature increased from 8°C to 14°C. The pond water was exchanged by continuous running water.

The base generation (G0) was established in 2006 and 2007 using hybridization between populations and artificial insemination. Lively individuals, which had no trauma and body weight of more than 1 kg, could be selected as parent fish. Full- and half-sib families were produced via an unbalanced nested mating design by randomly mating selected males and females from different populations. The numbers of turbot from different populations used to produce the base generation (G0) are shown in Table 1, and the numbers of family in each hybridized combination were shown in Table 2.

Table 1. Number of turbot from different introduced populations used to produce the base population

<table>
<thead>
<tr>
<th>Population type</th>
<th>Numbers for sires</th>
<th>Numbers for dams</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denmark</td>
<td>8</td>
<td>34</td>
</tr>
<tr>
<td>France</td>
<td>21</td>
<td>6</td>
</tr>
<tr>
<td>Britain</td>
<td>9</td>
<td>19</td>
</tr>
<tr>
<td>Spain</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>Chile</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 2. Number of families used to produce the base population from each cross combination

<table>
<thead>
<tr>
<th>Cross combinations (♀×♂)</th>
<th>Number of families</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denmark×Britain</td>
<td>4</td>
</tr>
<tr>
<td>Denmark×Chile</td>
<td>3</td>
</tr>
<tr>
<td>France×Denmark</td>
<td>32</td>
</tr>
<tr>
<td>France×Britain</td>
<td>15</td>
</tr>
<tr>
<td>France×Chile</td>
<td>5</td>
</tr>
<tr>
<td>Spain×Denmark</td>
<td>15</td>
</tr>
<tr>
<td>Spain×France</td>
<td>2</td>
</tr>
<tr>
<td>Spain×Britain</td>
<td>5</td>
</tr>
<tr>
<td>Spain×Chile</td>
<td>4</td>
</tr>
<tr>
<td>Britain×Denmark</td>
<td>12</td>
</tr>
<tr>
<td>Chile×Britain</td>
<td>5</td>
</tr>
<tr>
<td>Chile×Denmark</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>112</td>
</tr>
</tbody>
</table>

Fertilized eggs were incubated in mini flow water at 13.5–15°C until hatching (~3–5 d). All of the families were then reared separately in fiberglass-reinforced plastic tanks (0.5 m³) at a density of thirty thousand larvae (~50 mL fertilized eggs). The temperature of the larvae culture was maintained at 16–20°C. Salinity was 25–30 and dissolved oxygen was maintained at 4.9–8.0 mg/L. The rearing procedure was kept as consistent as possible for the different families. At the 35th and 70th days after hatching, 1 000 larvae and 400 juvenile fish per family were selected randomly and reared in new fiberglass-reinforced plastic tanks (0.5 m³). At the 3rd months after hatching, 80 individuals with a greater body weight than the mean from each family were selected randomly, weighed, and marked with a VIE tag. The fish were then randomly distributed into either one or several 5 m×5 m×0.6 m (L×W×H) test ponds in two farms. The VIE tag was updated once every three months. Approximately 15 months post-hatching, all of the surviving individuals were landed, towed dry and measured harvest body weight using electronic scale in turn. The progenies were genetically evaluated for the harvest body weight trait.

2.3 Production of selection generation (G1 and G2)

The subsequent generations (G1 and G2) were established by family and within-family selection strategy. From G0 to G2, all the full-sib families were ranked according their average family breeding values and about 40 families with high average family breeding values were selected as the parents of the next generation. Selected brood individuals were injected with passive integrated transponder (PIT) markers to distinguish them. Full- and half-sib families in G1 and G2 were produced via an unbalanced nested mating design by mating selected males and females from different families. Families with high average family breeding values were mated first and the candidate parents with higher breeding values were mated first. Brood individuals were mated randomly and inbreeding coefficient was maintained at ≤1%. Rearing and tagging of families in G1 and G2 were the same as G0.

2.4 Data analysis

Because we used PIT markers and VIE tags, a complete pedigree containing all phenotypically measured individuals was available and used in this study. The mixed model should include all effects that potentially played a role on the harvest body weight. The fixed effects considered in this study were year and farm. Each effect contains different factors that impact growth. The year effect contained annual breeding environment factors (e.g., water temperature and quality) and breeding operation. The farm effect contained raising environment, management level, stocking density, etc. A factor at different levels may have different effects on another factor, so their interaction was also included. The animal model was used to estimate the variance component using REML method with ASREML software (Gilmour et al., 2009) as follows:

\[ y_{ijkm} = \mu + a_i + F_j + Y_k + F_j \times Y_k + A_i (F_j \times Y_k) + d_m + e_{ijkm}, \]

where \( y_{ijkm} \) is the phenotypic observation for harvest body weight, \( \mu \) is the overall mean, \( a_i \) is the random additive genetic effect of animal \( i \), \( F_j \) is the fixed effect of farm \( j \) (two levels), \( Y_k \) is the fixed effect of year (seven levels), \( F_j \times Y_k \) is the interaction between farm and year, \( A_i (F_j \times Y_k) \) is a linear covariate nested within the interaction between year and farm, \( d_m \) is the random maternal and common environmental effects on full-sib \( m \) and \( e_{ijkm} \) is the random error term.

The distribution of the random effects \( a, d, \) and \( e \) were assumed to be normal, with means of zero. The variance-covariance matrix was as follows:
Table 3. The least squares means of harvest body weight in two farms by each year

<table>
<thead>
<tr>
<th>Year</th>
<th>Farm</th>
<th>Number of individuals</th>
<th>Least squares mean/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>2006</td>
<td>Haiyang</td>
<td>1 307</td>
<td>328.18</td>
</tr>
<tr>
<td></td>
<td>Rushan</td>
<td>*</td>
<td>–</td>
</tr>
<tr>
<td>2007</td>
<td>Haiyang</td>
<td>1 775</td>
<td>274.27</td>
</tr>
<tr>
<td></td>
<td>Rushan</td>
<td>*</td>
<td>–</td>
</tr>
<tr>
<td>2009</td>
<td>Haiyang</td>
<td>234</td>
<td>294.29</td>
</tr>
<tr>
<td></td>
<td>Rushan</td>
<td>761</td>
<td>337.36</td>
</tr>
<tr>
<td>2010</td>
<td>Haiyang</td>
<td>*</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Rushan</td>
<td>682</td>
<td>323.52</td>
</tr>
<tr>
<td>2011</td>
<td>Haiyang</td>
<td>1 305</td>
<td>336.86</td>
</tr>
<tr>
<td></td>
<td>Rushan</td>
<td>1 018</td>
<td>361.23</td>
</tr>
<tr>
<td>2013</td>
<td>Haiyang</td>
<td>1 946</td>
<td>332.42</td>
</tr>
<tr>
<td></td>
<td>Rushan</td>
<td>*</td>
<td>–</td>
</tr>
<tr>
<td>2014</td>
<td>Haiyang</td>
<td>*</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Rushan</td>
<td>1 924</td>
<td>347.35</td>
</tr>
</tbody>
</table>

Note: The asterisk (*) indicates turbot were not cultured in the farm.

There were no and very few mature female fish in 2008 and 2012, respectively, therefore, no families were produced in either year. Because the selected parent individuals develop at different rates, each generation contained families in either two or three years. The number of full-sib families for each year ranged from 54 to 97. Because some parent individuals were mated with more than one partner, there were also some maternal half-sib families and paternal half-sib families in each year. The data set consisted of 10 952 individuals in total. The average number of individuals in each full-sib family ranged from 12.63 to 31.14 in different years.

3.2 Genetic analysis

The variance components, heritability, and maternal and common environmental effects are listed in Table 5. Four heritability (h²) estimators for the harvest body weight were obtained. Heritability within G0 (0.11±0.08) was lower than that within G1, G2, and across generations. The other estimates were very similar, 0.18±0.09 within G1, 0.17±0.07 within G2, and 0.19±0.04 across generations. The heritability estimate for body weight in G0 was not significantly different from zero and in G1 and G2 were significantly different from zero. The heritability estimate across generations was very significantly different from zero. Maternal and common environmental effects (c²) within and across generations closely corresponded to heritability. The heritability and maternal and common environmental effects standard errors across generations were smaller than those within genera-

\[
V = \begin{bmatrix} a & 0 & 0 \\ d & Ia^2 & 0 \\ e & 0 & Ie^2 \\ 0 & 0 & Ic^2 \\ \end{bmatrix}
\]

where \( \sigma_a^2, \sigma_d^2, \) and \( \sigma_e^2 \) are the variance of the random effects \( a, d, \) and \( e, \) respectively. \( A \) is the \( n \times n \) matrix of additive genetic relationship, and \( I_a \) and \( I_e \) are \( n \times 1 \) identity matrices. \( n \) is the number of individuals.

Total phenotypic variance \( (\sigma_y^2) \) was calculated as the sum of the additive genetic variance \( (\sigma_a^2) \), the maternal and common environmental effects variance \( (\sigma_e^2) \), and the error variance \( (\sigma_e^2) \). The heritability \( (h^2) \) was computed as \( \sigma_a^2/\sigma_y^2 \), and maternal and common environmental effects \( (c^2) \) as \( \sigma_e^2/\sigma_y^2 \). All of the calculations were carried out in ASREML software (Gilmour et al., 2009).

The \( z \)-score was used to test whether the heritability significantly varied from zero:

\[
z = \frac{h^2}{se},
\]

where \( h^2 \) is the estimates of heritability, and \( se \) is the standard errors of heritability.

The selection differential for growth was estimated for each generation by comparing the mean estimated breeding value of the brooders that actually produced progeny and the mean of all of the fish before the brooders were selected. Estimated breeding values were calculated based on phenotype information available at the time of selection. The genetic gain in each generation was also calculated by comparing the mean estimated breeding value of the current and previous generation. The cumulative genetic gain after two selections was expressed as a percentage based on the following formula:

\[
P_c = \prod_{i=1}^{n} \left( 1 + P_i \right) - 1,
\]

where \( P_i \) is the cumulative genetic gain (%) for the \( i \)-th generation, and \( n \) is the number of the selection generation (\( i = 1, 2 \)).

3 Results

3.1 Descriptive statistics

The main statistical results of data set are shown in Table 3 and Table 4. The least square means of harvest body weight in Rushan farm were larger than Haiyang in 2009 and 2011 and progenies in the other years were only cultured in one farm. In total, 508 full-sib turbot families were constructed from 2006 to 2014.

Table 4. Number of sire, dam, families, individuals, average number of individuals per family, and least squares mean in each year and generation

<table>
<thead>
<tr>
<th>Generation</th>
<th>Year</th>
<th>Sire</th>
<th>Dam</th>
<th>Full-sib families</th>
<th>Sire half-sib families</th>
<th>Dam half-sib families</th>
<th>Number of individuals</th>
<th>Average number of individuals per family</th>
<th>Least squares mean/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0</td>
<td>2006</td>
<td>32</td>
<td>33</td>
<td>55</td>
<td>19</td>
<td>14</td>
<td>1 307</td>
<td>23.76</td>
<td>299.250</td>
</tr>
<tr>
<td></td>
<td>2007</td>
<td>46</td>
<td>33</td>
<td>57</td>
<td>14</td>
<td>10</td>
<td>1 775</td>
<td>31.14</td>
<td>347.070</td>
</tr>
<tr>
<td>G1</td>
<td>2009</td>
<td>46</td>
<td>37</td>
<td>71</td>
<td>17</td>
<td>18</td>
<td>995</td>
<td>14.01</td>
<td>338.891</td>
</tr>
<tr>
<td></td>
<td>2010</td>
<td>44</td>
<td>26</td>
<td>54</td>
<td>9</td>
<td>12</td>
<td>682</td>
<td>12.63</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>75</td>
<td>49</td>
<td>95</td>
<td>18</td>
<td>27</td>
<td>323</td>
<td>24.45</td>
<td>–</td>
</tr>
<tr>
<td>G2</td>
<td>2013</td>
<td>53</td>
<td>35</td>
<td>79</td>
<td>17</td>
<td>17</td>
<td>1 946</td>
<td>24.63</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2014</td>
<td>80</td>
<td>52</td>
<td>97</td>
<td>13</td>
<td>29</td>
<td>1 924</td>
<td>19.84</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>376</td>
<td>265</td>
<td>508</td>
<td>107</td>
<td>63</td>
<td>376</td>
<td>10 952</td>
<td>21.56</td>
<td>–</td>
</tr>
</tbody>
</table>
might increase resemblance in family members. The heritability resulted in a slight bias in the heritability estimate, because it more, selecting individuals 3 months post-hatching may have possible subsistent fixed effect of pond was ignored. Further-
ated and phenotypic variance was overestimated because some estimators (Gall et al., 1993); the heritability estimated in this onmental conditions, and statistical models all result in different sific populations and environments, different populations, envir-
ence was not significant in the early stage.

Wang et al. (2014) also obtained similar results, although female Furthermore, Lyu et al. (2016) reported that variance analysis re-
phologically at 15 months old and, moreover, dissecting to ob-
average of 19.72 g per generation. The genetic gains were also obtained, they were 22.06 g in G1 and 11.93 g in G2, corresponding to 6.36% and 3.52% body weight. The cumulative genetic gain after two generations was 10.10% body weight.

### 4 Discussion

#### 4.1 Genetic analysis

In this study, sex was not included in the mixed effect model analysis, because it is difficult to determine turbot gender morphologically at 15 months old and, moreover, dissecting to observe the gonad inevitably results in the death of the individual. Furthermore, Lyu et al. (2016) reported that variance analysis revealed no significant fixed effects of sex on harvest body weight. Wang et al. (2014) also obtained similar results, although female turbot had a growth advantage compared with males the difference was not significant in the early stage.

Heritability estimates for body weight within and across generations were all low to medium in 15 month-old turbot, based on the following categorization: low (0.05–0.15), medium (0.20–0.40), high (0.45–0.60), and very high (>0.65) (Cardellino and Rovira, 1987). There is a limited number of publications regarding the genetic parameters for body weight traits in turbot. Most previous estimates for body weight of adult turbot were medium to high (Gjerde et al., 1997; Ma et al., 2006; Lyu et al., 2016). Although genetic parameter estimates are only applicable to specific populations and environments, different populations, environmental conditions, and statistical models all result in different estimators (Gall et al., 1993); the heritability estimated in this study was lower than previous reports, which suggests that our heritability values may be underestimated. The most likely reason is that the sum of fixed environment effects was underestimated and phenotypic variance was overestimated because some possible subsistent fixed effect of pond was ignored. Furthermore, selecting individuals 3 months post-hatching may have resulted in a slight bias in the heritability estimate, because it might increase resemblance in family members. The heritability values for harvest body weight remained stable across genera-

### Table 5. Variance components, heritability, and maternal and common environmental effects on harvest body weight within and across generations

<table>
<thead>
<tr>
<th>Generation</th>
<th>$\sigma^2_{p}$</th>
<th>$\sigma^2_{q}$</th>
<th>$\sigma^2_{e}$</th>
<th>$\sigma^2_{l}$</th>
<th>$h^2 \pm se$</th>
<th>$c^2 \pm se$</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0</td>
<td>21 725.00</td>
<td>2 442.73</td>
<td>2 171.65</td>
<td>17 100.90</td>
<td>0.11±0.08</td>
<td>0.16±0.04</td>
</tr>
<tr>
<td>G1</td>
<td>14 690.42</td>
<td>2 789.38</td>
<td>2 209.84</td>
<td>10 291.20</td>
<td>0.18±0.09*</td>
<td>0.14±0.04</td>
</tr>
<tr>
<td>G2</td>
<td>7 054.70</td>
<td>1 182.06</td>
<td>935.41</td>
<td>4 937.14</td>
<td>0.17±0.07*</td>
<td>0.13±0.03</td>
</tr>
<tr>
<td>Across-gen</td>
<td>17 156.17</td>
<td>3 289.37</td>
<td>2 099.40</td>
<td>11 767.40</td>
<td>0.19±0.04**</td>
<td>0.12±0.01</td>
</tr>
</tbody>
</table>

Note: The asterisk (*) indicates estimate is significantly different from zero ($P<0.05$) and the double-asterisk (**) estimate is very significantly different from zero ($P<0.01$).

### Table 6. Average estimated breeding value, average estimated breeding value of brooders, selection differential, and genetic gain in each generation

<table>
<thead>
<tr>
<th>Generation</th>
<th>Average estimated breeding value of all individuals/g</th>
<th>Average estimated breeding value of brooders/g</th>
<th>Selection differential/g</th>
<th>Genetic gain/g</th>
<th>Genetic gain/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0</td>
<td>-0.82</td>
<td>17.42</td>
<td>18.24</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G1</td>
<td>21.24</td>
<td>42.43</td>
<td>21.19</td>
<td>22.06</td>
<td>6.36</td>
</tr>
<tr>
<td>G2</td>
<td>33.17</td>
<td>-</td>
<td>-</td>
<td>11.93</td>
<td>3.52</td>
</tr>
</tbody>
</table>

### 3.3 Response to selection

The average estimated breeding value of all individuals and brooders in each generation are listed in Table 6. The growth selection differential was 18.24 g in G0 and 21.19 g in G1 corresponding to an average of 19.72 g per generation. The genetic gains were obtained, they were 22.06 g in G1 and 11.93 g in G2, corresponding to 6.36% and 3.52% body weight. The cumulative genetic gain after two generations was 10.10% body weight.
al phenotypic change should only correspond to the expected response to selection when the environmental effects on the parental and progeny generations are identical (Gall et al., 1993).

After two generation selection, both average estimated breeding and phenotypic variances were improved, which indicated that the BLUP selective breeding program for the body weight trait in turbot was successful. Nonetheless, the average genetic gains in this study were relatively small (<10% in one generation), compared with earlier reports on other aquatic species (Charo-Karisa et al., 2006; Liu et al., 2014; Luan et al., 2012; Maluwa and Gjerde, 2007; Rezk et al., 2009; Thodesen et al., 2012). Gjedrem (2000) also reported that genetic gain in growth per generation for aquatic animal species ranged from 10% to 20%. The main reason for low genetic gain in this study is that the proportion of turbot with well-developed gonads was low, which resulted in a low selection intensity.

In this study, the number of full-sib families for each year had a large variation, ranging from 54 to 97, and also there were significant differences among at least square means of harvest body weight by each year. These results suggested aquaculture environment and management situations were far from the best condition, especially in gonad development process. Number of cultured families was restricted by a low proportion of turbot with well-developed gonads (especially in female). A sufficient number of full-sib families form the basis of increasing selection intensity and avoiding inbreeding, which results in phenotypic depression (Thodesen et al., 2005).

References


Unravelling habitat use of *Coilia nasus* from the Rokkaku River and Chikugo River estuaries of Japan by otolith strontium and calcium

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**Abstract**

The migratory history of the engraulid fish *Coilia nasus* in the Rokkaku and Chikugo River estuaries of the Ariake Sea, Japan was assessed using otolith strontium (Sr) X-ray intensity maps and strontium:calcium (Sr:Ca) ratio life history transects by an electron probe microanalyzer (EPMA). The results showed that seven of the ten specimens from the Rokkaku River Estuary (LJC) and all 15 specimens collected in the Chikugo River Estuary (ZHC) had low Sr:Ca ratios (<3) at the central otolith area, indicating their riverine origin and initial freshwater residence. After the first regime shift adjacent to natal regions, the Sr level mapping displayed a wide variety of color patterns, and the Sr:Ca ratios obtained by the line transect analysis could be divided into one to six significantly different phases indicative of gradual life history transition. The other three specimens from the Rokkaku River Estuary had high Sr:Ca ratios (3–6.7) at the central otolith area but showed alternating changes between low and high values outside the natal region, suggesting that estuarine-origin individuals occurred in the Rokkaku River Estuary. The two-dimensional maps of the Sr level and average of the otolith Sr:Ca ratios along the life-history transects could be used as effective tools for reconstruction of past habitat use of the tapertail anchovy in estuaries of the Ariake Sea, Japan.

**Key words:** *Coilia nasus*, otolith microchemistry, habitat use, Rokkaku River, Chikugo River


1 **Introduction**

The estuarine tapertail anchovy *Coilia nasus* Temminck et Schlegel, 1846 (junior synonym *Coilia ectenes* Jordan et Seale, 1905) is commonly distributed in East Asia including China, Korea, and Japan. *Coilia nasus* is a famous traditional delicacy, and is a highly commercial anadromous species present in the estuaries of several main rivers (e.g., Changjiang River (Yangtze River), Huanghe River (Yellow River), Qiantangjiang River, Oujiang River) in China (Jiang et al., 2014), and the estuaries of rivers (especially the Chikugo River) flowing into the Ariake Sea of Kyushu in Japan (Suzuki et al., 2014). As *C. nasus* is an endemic and restricted species in the Ariake areas of Japan, it has been listed as a threatened species in the Japan Red Data Book of Japan’s Ministry of the Environment and placed in the category of “vulnerable” species by Japan’s Fisheries Agency. Importantly, this species is regarded from its habitat as an Asian continental relict (Yagi et al., 2011; Simanjuntak et al., 2015) in Japan, as only the Ariake Sea retained an environment of large tidal range and vast tidal flats similar to those in the Yellow Sea and East China Sea (Yagi et al., 2011). Therefore, life histories between the *C. nasus* populations in China (e.g., the Changjiang River) and Japan (e.g., the Chikugo River) may be comparable.

So far, there have been a considerable number of studies on distribution patterns, feeding habits and spawning areas of *C. nasus* in the estuary of the Chikugo River, which is the largest river discharging into the Ariake Sea (e.g., Takita and Masutani, 1979; Matsu, 1986a, b; Suzuki et al., 2014; Simanjuntak et al., 2015). However, the spatial and temporal dynamics are still poorly known for this species during the life history in the estuaries of other rivers (e.g., the Rokkaku River) along the coast of the Ariake Sea. Noteworthy, otoliths of teleost fish grow continuously throughout entire life history (Campana, 1999) and otolith core region corresponds to the larval period (Secor et al., 1998). Moreover, previous studies have already demonstrated that otolith strontium (Sr) and calcium (Ca) signatures can be applied as a useful scalar to trace environmental history of fish, since the deposition of Sr and the Sr:Ca ratio positively correlates with the salinities of freshwater, brackish water, and sea water (Secor and Rooker, 2000; Zimmerman, 2005; Yang et al., 2011). Recently, based on otolith microchemical analysis of Sr:Ca ratio and Sr content, a number of previously unknown features of life history patterns were revealed in *C. nasus* from the Changjiang River in China. These include the composition of an apparent spawning population by individuals with a variety of migratory patterns, es-

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tuarine or freshwater resident phenotypes of *C. nasus*, and the tendency of different natal riverine homing of *C. nasus* in different water areas along the Chinese coast (Yang et al., 2006; Dou et al., 2012; Jiang et al., 2014, 2016; Chen et al., 2017). These updated studies provide more accurate and detailed evidence for the need for further assessment of quantity and resource composition of the anchovies with different migratory patterns, as well as the need to develop a plan of habitat conservation and nature reserve settlement. Furthermore, an improved understanding of diverse mechanisms to adapt to different salinity environment of this fish in Japan and China is desirable. Therefore, we hypothesized that *C. nasus* in river estuaries around the Ariake Sea of Japan must have much more diverse and flexible patterns of life history among habitats of fresh, brackish and sea water. Otolith microchemical analysis can give new insights to the ecological study and conservation of this endemic and continental relict species. To test the above hypothesis, in this pilot study we examined the otolith Sr and Ca microchemistry of *C. nasus* in the estuaries of the Chikugo River and the Rokkaku River.

The tapertail anchovy has been confirmed to inhabit both the Chikugo River and Rokkaku River although the spawning sites may only be located in the Chikugo River (Takita and Masutani, 1979). The Rokkaku River Estuary is a brackish, highly turbid water estuary which is located in the northern portion of the Ariake Sea (Yagi et al., 2011), while the Chikugo River is the largest river flowing into the Ariake Sea which receives strong tidal currents in the north-eastern part of the inner Ariake Bay (Islam et al., 2007; Suzuki et al., 2014).

The objectives of the present study were to validate the environmental markers of Sr and Ca in the otoliths of *C. nasus* from the estuaries of the Rokkaku River and Chikugo River around the Ariake Sea and accumulate information regarding the spatial and temporal dynamics in habitat use of the *C. nasus*. The information will be important for effective conservation and management of the valuable species.

2 Materials and methods

The target field sites of this study are the Rokkaku River and Chikugo River estuaries (Fig. 1). Table 1 provides the background information of *C. nasus* collected from different regions.

Ten specimens of *C. nasus* (total length (*L*<sub>T</sub>): range, 15.7–23.8 cm; mean±SD, (20.2±2.3) cm; similarly hereafter) aged 1–2 years were captured in the Rokkaku River Estuary (LJC) in Saga Prefecture on 15 June and 30 August of 2011. The other fifteen *C. nasus* (*L*<sub>T</sub>: 26.3–32.5 cm, (29.2±1.8) cm) aged 2–3 years were collected in the Jojima section of the Chikugo River (ZHIC) in Fukuoka Prefecture on 30 June of 2015.

Methods of preparing *C. nasus* otoliths for use in electron probe microanalysis (EPMA) measurement were described by Jiang et al. (2017) and Chen et al. (2017). The sagittae otoliths were extracted first. After cleaning with deionized water, all left otoliths were embedded in epoxy resin (EpoFix; Struers, Copenhagen, Denmark) in the frontal plane until solidification. The embedded otoliths were ground to expose their core with a grinding machine equipped with a diamond cup wheel (Discoplan-TS; Struers, Copenhagen, Denmark). Each otolith was further polished on an automated polishing wheel (LaboPol-35, Struers, Copenhagen, Denmark). The otoliths were then cleaned in an ultrasonic bath and rinsed with deionized water. Finally, all otoliths were dried and then carbon coated by a high vacuum evaporator (JEE-420, JEOL Ltd., Tokyo, Japan) for further examination.

EPMA was used to study the otolith elements Sr and Ca, based on the method described by Chen et al. (2017) but with a slight modification. Samples were measured along a line of elements detectable in each otolith from the core to the edge (i.e., otolith radius) using a wavelength dispersive X-ray electron probe microanalyzer (JXA-8100; JEOL Ltd.). Commercial standards of tautonite (SrTiO<sub>3</sub>) and calcite (CaCO<sub>3</sub>) (Institute of Mineral Resources, the Chinese Academy of Geological Sciences, Beijing, China) were used for calibrating the Sr and Ca contents in the otolith samples. The accelerating voltage and beam current were 15 kV and 2×10<sup>–8</sup> A, respectively. The electron beam probe microanalysis (EPMA) measurement were described by Jiang et al. (2017) and Chen et al. (2017). The sagittae otoliths were extracted first. After cleaning with deionized water, all left otoliths were embedded in epoxy resin (EpoFix; Struers, Copenhagen, Denmark) in the frontal plane until solidification. The embedded otoliths were ground to expose their core with a grinding machine equipped with a diamond cup wheel (Discoplan-TS; Struers, Copenhagen, Denmark). Each otolith was further polished on an automated polishing wheel (LaboPol-35, Struers, Copenhagen, Denmark). The otoliths were then cleaned in an ultrasonic bath and rinsed with deionized water. Finally, all otoliths were dried and then carbon coated by a high vacuum evaporator (JEE-420, JEOL Ltd., Tokyo, Japan) for further examination.

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By conventional otolith research, Sr:Ca ratios of concentrations were expressed as the ratio of Sr to Ca amplifying by 1000 by a simple conversion based on the molecular weights of SrO and CaO. Statistical analysis was performed using Excel 2013 (Microsoft, Seattle, WA, USA) and IBM SPSS Statistics v.19.0 (IBM Corp., Armonk, NY, USA). The Mann-Whitney U-test was used to test the differences between otolith Sr:Ca ratios. In addition, a sequential regime shift algorithm was applied to identify significant changes between the current mean of Sr:Ca ratio data and that of a consecutive point, an indication of the life history transition (Rodionov, 2004; Altenritter et al., 2015). The inputs of the regime shift index (RSI) were established as follows: cut-off length=10, probability level=0.1, and Huber’s weight parameter=1 (Rodionov and Overland, 2005).

3 Results

3.1 *Coilia nasus* from the Rokkaku River

In the ten specimens of *C. nasus* from the Rokkaku River Estuary, two patterns of otolith in the life history transect were observed (Table 2, Fig. 2). In the first pattern, the Sr:Ca ratios in the central regions of seven fish (LJC01, LJC02, LJC05, LJC07, LJC08, LJC09, and LJC10) otoliths possessed low values (1.98±0.91 to 2.75±0.82, similarly hereafter) within a distance of 50–1630 μm from the otolith core, probably corresponding to larval (LJC01, LJC02, LJC07, LJC08, LJC09, and LJC10) stages. The X-ray intensity map of Sr in the remaining otoliths displayed a wide variety of color patterns (from blue-lowest, to green to red-highest, Fig. 3). The Sr:Ca ratios obtained by the line transect analysis could be divided into one (LJC07 and LJC08) to five (LJC05) significantly different phases indicative of...
life history transition based on the aforementioned sequential regime shift algorithm (Table 2, Fig. 2). Particularly, mean values of the Sr:Ca ratios could be as high as >7 in certain areas of the otolith regions in the individuals of LJC01, LJC02, LJC09 (Table 2) and generally remained at high levels (>4.50) in the other regions.

In the second pattern, the Sr:Ca ratios in the central regions of three fish (LJC03, LJC04, and LJC06) otoliths had high values (>3, 3.23±0.72 to 5.62±1.68) within a distance of 90–420 μm from the otolith core, although those in the other regions varied drastically at low (2.61±0.61 to 2.68±0.70) or high levels (4.60±1.10 to 7.77±0.90) (Table 2, Fig. 2). The X-ray intensity maps of the Sr-level distribution in the otoliths showed patterns similar to those of the Sr:Ca ratios in the above life history transects with a wide variety of color patterns (Fig. 3).

Generally, all otolith samples of LJC01, LJC02, LJC05, LJC07, LJC08, LJC09, and LJC10 had similar bluish central regions, suggesting that the fish were born in freshwater. Thereafter, the color patterns alternated among greenish or yellowish or even reddish bands, corresponding to the migration of these fish in the estuarine and even offshore sea habitats (e.g., LJC01, LJC02, and LJC09) (Fig. 3). In contrast, otolith samples of LJC03, LJC04, and LJC06 had greenish or yellowish central regions. The color patterns for the other regions of these fish otoliths varied among bluish, greenish, yellowish or reddish bands, which implied that the fish were not born in freshwater but were more likely born in estuarine brackish water.

### Table 2. Fluctuation of Sr and Ca microchemistry in otoliths of *Coilia nasus* from the Rokkaku River Estuary, Kyushu, Japan

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Distance from the core/μm</th>
<th>Sr:Ca ratio</th>
<th>Fluctuation phases of otolith Sr:Ca ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>LJC01</td>
<td>0–270</td>
<td>2.75±0.82</td>
<td>1a (FW)</td>
</tr>
<tr>
<td></td>
<td>270–890</td>
<td>5.41±1.08</td>
<td>2a (BW)</td>
</tr>
<tr>
<td></td>
<td>890–1 130</td>
<td>7.13±0.74</td>
<td>3a (SW)</td>
</tr>
<tr>
<td></td>
<td>1 130–1 560</td>
<td>5.06±0.72</td>
<td>4a (BW)</td>
</tr>
<tr>
<td>LJC02</td>
<td>0–50</td>
<td>1.98±0.91</td>
<td>1a (FW)</td>
</tr>
<tr>
<td></td>
<td>50–380</td>
<td>5.54±1.22</td>
<td>2a (BW)</td>
</tr>
<tr>
<td></td>
<td>380–1 190</td>
<td>8.38±0.89</td>
<td>3a (SW)</td>
</tr>
<tr>
<td></td>
<td>1 190–1 780</td>
<td>5.5±1.45</td>
<td>4a (BW)</td>
</tr>
<tr>
<td>LJC03</td>
<td>0–100</td>
<td>5.62±1.68</td>
<td>1a (BW)</td>
</tr>
<tr>
<td></td>
<td>100–900</td>
<td>7.77±0.9</td>
<td>2a (SW)</td>
</tr>
<tr>
<td></td>
<td>900–1 680</td>
<td>5.13±1.32</td>
<td>3a (BW)</td>
</tr>
<tr>
<td>LJC04</td>
<td>0–90</td>
<td>4.84±1.49</td>
<td>1a (BW)</td>
</tr>
<tr>
<td></td>
<td>90–220</td>
<td>2.61±0.61</td>
<td>2a (FW)</td>
</tr>
<tr>
<td></td>
<td>220–510</td>
<td>4.6±1.1</td>
<td>3a (BW)</td>
</tr>
<tr>
<td></td>
<td>510–1 040</td>
<td>7.71±0.92</td>
<td>4a (SW)</td>
</tr>
<tr>
<td></td>
<td>1 040–1 600</td>
<td>4.88±0.86</td>
<td>5a (BW)</td>
</tr>
<tr>
<td>LJC05</td>
<td>0–180</td>
<td>2.3±0.76</td>
<td>1a (FW)</td>
</tr>
<tr>
<td></td>
<td>180–330</td>
<td>3.57±0.73</td>
<td>2a (BW)</td>
</tr>
<tr>
<td></td>
<td>330–420</td>
<td>2.4±0.73</td>
<td>3a (FW)</td>
</tr>
<tr>
<td></td>
<td>420–520</td>
<td>4.66±0.55</td>
<td>4a (BW)</td>
</tr>
<tr>
<td></td>
<td>520–1 060</td>
<td>2.53±0.78</td>
<td>5a (FW)</td>
</tr>
<tr>
<td></td>
<td>1 060–1 360</td>
<td>5.8±0.85</td>
<td>6a (BW)</td>
</tr>
<tr>
<td>LJC06</td>
<td>0–420</td>
<td>3.23±0.72</td>
<td>1a (BW)</td>
</tr>
<tr>
<td></td>
<td>420–1 190</td>
<td>2.68±0.7</td>
<td>2a (FW)</td>
</tr>
<tr>
<td></td>
<td>1 190–1 480</td>
<td>5.9±0.92</td>
<td>3a (BW)</td>
</tr>
<tr>
<td>LJC07</td>
<td>0–1 200</td>
<td>2.18±0.9</td>
<td>1a (FW)</td>
</tr>
<tr>
<td></td>
<td>1 200–1 770</td>
<td>4.37±1.18</td>
<td>2a (BW)</td>
</tr>
<tr>
<td>LJC08</td>
<td>0–1 630</td>
<td>2.11±0.74</td>
<td>1a (FW)</td>
</tr>
<tr>
<td></td>
<td>1 630–1 840</td>
<td>4.55±0.98</td>
<td>2a (BW)</td>
</tr>
<tr>
<td>LJC09</td>
<td>0–250</td>
<td>2.44±0.83</td>
<td>1a (FW)</td>
</tr>
<tr>
<td></td>
<td>250–410</td>
<td>4.5±0.96</td>
<td>2a (BW)</td>
</tr>
<tr>
<td></td>
<td>410–700</td>
<td>7.06±1.13</td>
<td>3a (SW)</td>
</tr>
<tr>
<td></td>
<td>700–1 870</td>
<td>4.6±1.27</td>
<td>4a (BW)</td>
</tr>
<tr>
<td>LJC10</td>
<td>0–980</td>
<td>2.24±0.81</td>
<td>1a (FW)</td>
</tr>
<tr>
<td></td>
<td>980–1 340</td>
<td>3.58±0.78</td>
<td>2a (BW)</td>
</tr>
<tr>
<td></td>
<td>1 340–1 570</td>
<td>2.84±0.94</td>
<td>3a (FW)</td>
</tr>
</tbody>
</table>

Note: Sr:Ca ratio is expressed as the ratio of Sr to Ca amplifying by 1 000. Phases in one otolith sample having the same superscript letter indicate no significant differences ($P>0.05$); whereas different superscript letters indicate significant differences ($P<0.05$). FW, BW and SW indicate the phases of freshwater, brackish water and sea water, respectively.

In the second pattern, the Sr:Ca ratios in the central regions of three fish (LJC03, LJC04, and LJC06) otoliths had high values (>3, 3.23±0.72 to 5.62±1.68) within a distance of 90–420 μm from the otolith core, although those in the other regions varied drastically at low (2.61±0.61 to 2.68±0.70) or high levels (4.60±1.10 to 7.77±0.90) (Table 2, Fig. 2). The X-ray intensity maps of the Sr-level distribution in the otoliths showed patterns similar to those of the Sr:Ca ratios in the above life history transects with a wide variety of color patterns (Fig. 3).

Generally, all otolith samples of LJC01, LJC02, LJC05, LJC07, LJC08, LJC09, and LJC10 had similar bluish central regions, suggesting that the fish were born in freshwater. Thereafter, the color patterns alternated among greenish or yellowish or even reddish bands, corresponding to the migration of these fish in the estuarine and even offshore sea habitats (e.g., LJC01, LJC02, and LJC09) (Fig. 3). In contrast, otolith samples of LJC03, LJC04, and LJC06 had greenish or yellowish central regions. The color patterns for the other regions of these fish otoliths varied among bluish, greenish, yellowish or reddish bands, which implied that the fish were not born in freshwater but were more likely born in estuarine brackish water.

### 3.2 Coilia nasus from the Chikugo River

In the fifteen specimens of *C. nasus* captured from the Chikugo River Estuary, the life-history transect analyses (Table 3, Fig. 4) and X-ray intensity maps (Fig. 5) showed that all otoliths had a similar bluish color patterns and low Sr:Ca ratios (<3) in their central regions, which were consistent with a presumed...
### Table 3. Fluctuation of Sr and Ca microchemistry in otoliths of *Coilia nasus* from Jojima section of the Chikugo River, Kyushu, Japan

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Distance from the core/µm</th>
<th>Sr:Ca ratio</th>
<th>Fluctuation phases of otolith Sr:Ca ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZHC01</td>
<td>0–80</td>
<td>1.97±0.79</td>
<td>1&lt;sup&gt;a&lt;/sup&gt; (FW)</td>
</tr>
<tr>
<td></td>
<td>80–790</td>
<td>3.68±0.86</td>
<td>2&lt;sup&gt;b&lt;/sup&gt; (BW)</td>
</tr>
<tr>
<td></td>
<td>790–1 010</td>
<td>2.61±0.52</td>
<td>3&lt;sup&gt;c&lt;/sup&gt; (FW)</td>
</tr>
<tr>
<td></td>
<td>1 010–1 780</td>
<td>4.12±0.98</td>
<td>4&lt;sup&gt;d&lt;/sup&gt; (BW)</td>
</tr>
<tr>
<td></td>
<td>1 780–1 970</td>
<td>2.2±0.8</td>
<td>5&lt;sup&gt;e&lt;/sup&gt; (FW)</td>
</tr>
<tr>
<td></td>
<td>1 970–2 060</td>
<td>3.68±1.02</td>
<td>6&lt;sup&gt;f&lt;/sup&gt; (BW)</td>
</tr>
<tr>
<td>ZHC02</td>
<td>0–230</td>
<td>1.68±0.57</td>
<td>1&lt;sup&gt;a&lt;/sup&gt; (FW)</td>
</tr>
<tr>
<td></td>
<td>230–530</td>
<td>3.42±0.92</td>
<td>2&lt;sup&gt;b&lt;/sup&gt; (BW)</td>
</tr>
<tr>
<td></td>
<td>530–710</td>
<td>2.67±0.74</td>
<td>3&lt;sup&gt;c&lt;/sup&gt; (FW)</td>
</tr>
<tr>
<td></td>
<td>710–1 390</td>
<td>4.68±0.92</td>
<td>4&lt;sup&gt;d&lt;/sup&gt; (BW)</td>
</tr>
<tr>
<td></td>
<td>0–130</td>
<td>1.66±0.57</td>
<td>1&lt;sup&gt;a&lt;/sup&gt; (FW)</td>
</tr>
<tr>
<td></td>
<td>130–370</td>
<td>3.48±1.04</td>
<td>2&lt;sup&gt;b&lt;/sup&gt; (BW)</td>
</tr>
<tr>
<td></td>
<td>370–1 030</td>
<td>2.41±0.99</td>
<td>3&lt;sup&gt;c&lt;/sup&gt; (FW)</td>
</tr>
<tr>
<td></td>
<td>1 030–1 290</td>
<td>3.59±0.73</td>
<td>4&lt;sup&gt;d&lt;/sup&gt; (BW)</td>
</tr>
<tr>
<td></td>
<td>1 290–1 340</td>
<td>1.93±0.5</td>
<td>5&lt;sup&gt;e&lt;/sup&gt; (FW)</td>
</tr>
<tr>
<td>ZHC03</td>
<td>0–800</td>
<td>2.24±0.79</td>
<td>1&lt;sup&gt;a&lt;/sup&gt; (FW)</td>
</tr>
<tr>
<td></td>
<td>800–1 400</td>
<td>3.88±0.8</td>
<td>2&lt;sup&gt;b&lt;/sup&gt; (BW)</td>
</tr>
<tr>
<td></td>
<td>1 400–1 740</td>
<td>2.69±0.66</td>
<td>3&lt;sup&gt;c&lt;/sup&gt; (FW)</td>
</tr>
<tr>
<td></td>
<td>0–430</td>
<td>1.95±0.78</td>
<td>1&lt;sup&gt;a&lt;/sup&gt; (FW)</td>
</tr>
<tr>
<td></td>
<td>440–490</td>
<td>3.88±1.27</td>
<td>2&lt;sup&gt;b&lt;/sup&gt; (BW)</td>
</tr>
<tr>
<td></td>
<td>500–590</td>
<td>7.35±0.6</td>
<td>3&lt;sup&gt;c&lt;/sup&gt; (SW)</td>
</tr>
<tr>
<td></td>
<td>590–1 350</td>
<td>4.68±1.36</td>
<td>4&lt;sup&gt;d&lt;/sup&gt; (BW)</td>
</tr>
<tr>
<td></td>
<td>1 350–1 450</td>
<td>2.05±0.63</td>
<td>5&lt;sup&gt;e&lt;/sup&gt; (FW)</td>
</tr>
<tr>
<td></td>
<td>1 450–1 550</td>
<td>3.48±0.42</td>
<td>6&lt;sup&gt;f&lt;/sup&gt; (BW)</td>
</tr>
<tr>
<td></td>
<td>1 550–1 670</td>
<td>2.46±1.02</td>
<td>7&lt;sup&gt;g&lt;/sup&gt; (FW)</td>
</tr>
<tr>
<td>ZHC05</td>
<td>0–230</td>
<td>2.05±0.6</td>
<td>1&lt;sup&gt;a&lt;/sup&gt; (FW)</td>
</tr>
<tr>
<td></td>
<td>230–1 490</td>
<td>4.07±1.19</td>
<td>2&lt;sup&gt;b&lt;/sup&gt; (BW)</td>
</tr>
<tr>
<td>ZHC06</td>
<td>0–200</td>
<td>2.29±1.05</td>
<td>1&lt;sup&gt;a&lt;/sup&gt; (FW)</td>
</tr>
<tr>
<td></td>
<td>200–440</td>
<td>4.81±1.14</td>
<td>2&lt;sup&gt;b&lt;/sup&gt; (BW)</td>
</tr>
<tr>
<td></td>
<td>440–530</td>
<td>6.28±0.66</td>
<td>3&lt;sup&gt;c&lt;/sup&gt; (BW)</td>
</tr>
<tr>
<td></td>
<td>530–1 140</td>
<td>4.96±1.05</td>
<td>4&lt;sup&gt;d&lt;/sup&gt; (BW)</td>
</tr>
<tr>
<td></td>
<td>1 140–1 710</td>
<td>2.58±0.8</td>
<td>5&lt;sup&gt;g&lt;/sup&gt; (FW)</td>
</tr>
</tbody>
</table>

Fig. 3. Strontium (Sr) imaging of *Coilia nasus* collected from the Rokkaku River Estuary using otolith mapping analysis.
freshwater residency period. Adjacent to the bluish regions in the center, patterns in Sr:Ca ratios along microprobe profiles varied among individuals. The concentric rings in the other regions of the otoliths varied from being bluish–greenish (ZHC01, ZHC02, ZHC03, ZHC04, ZHC14, ZHC15, ZHC17 and ZHC20) to bluish–greenish–yellowish–reddish (ZHC05, ZHC06, ZHC07, ZHC09, ZHC10, ZHC12 and ZHC19), which corresponded to the Sr:Ca ratios (∼1.5, mapping color: blue; 3–6.7, green or yellow; >6.7, red; Fig. 5). Mean Sr:Ca values observed were significantly different between two subsequent phases of Sr:Ca ratio and this phenomenon should be associated with transitions between water sources of different salinity (Table 3, Fig. 4). Interestingly, no high levels of the Sr:Ca ratios could be found in the central regions of the otoliths from all Chikugo C. nasus captured, unlike the situation observed in aforementioned Rokkaku C. nasus.

4 Discussion

Coilia nasus is traditionally believed to be one species of anadromous fish in Japan and China. During a long spawning season ranging from May to August, adults ascend to the river from the sea for spawning (Whitehead et al., 1988). Reproduction of the C. nasus population appears to greatly depend on the oligohaline region of the estuaries as the eggs and larvae of this fish might have a much better chance to survive in waters with very low salinity (Takita and Masutani, 1979; Suzuki et al., 2014), i.e., freshwater habitat. However, fluctuation (gray line) of Sr:Ca ratios along line transects from the core (0 μm) to the edge (Table 2, Fig. 2) and Sr imaging using otolith mapping analysis of sagital otoliths (Fig. 3) indicated that the habitat use of Rokkaku C. nasus was much more variable and complex than previously thought. Although the first pattern of otolith microchemistry in seven fish (LJC01, LJC02, LJC05, LJC07, LJC08, LJC09, and LJC10) showed a typical freshwater origin or anadromous pattern with obviously different phases of low-center high-edge of Sr:Ca ratio and Sr content map, the second pattern in the otoliths of three fish (LJC03, LJC04 and LJC06) with a high center region of Sr:Ca ratio (>3) and Sr content map (greenish or yellowish color) suggested that not all of the C. nasus anadromously migrated to the freshwater area to spawn. These results suggest that these individuals might all be of estuarine origin based on our previous studies of the otolithic salinity markers of freshwater, brackish water and seawater habitats by Sr:Ca ratios of wild C. nasus (Yang et al., 2006, 2011; Jiang et al., 2014). Unfortunately, it is still impossible to know the exact salinity requirement for the spawning sites of C. nasus in the Rokkaku River, due to a lack of corresponding studies so far. Although the eggs and larvae of C. nasus
originating from the Chikugo River were believed to be highly vulnerable to high-salinity brackish water (Takita and Masutani, 1979; Suzuki et al., 2014), this phenomenon will not be fully correct in those from the Rokkaku River. The otolith microchemical results of LJC03, LJC04 and LJC06 in the present study suggested that sporadic reproduction of *C. nasus* in the mesohaline zone of the Rokkaku River Estuary might be not impossible, i.e., some individuals could tolerate saltwater during early life stages (e.g.,

Fig. 4. Fluctuation (grey line) and shift (black line) of Sr:Ca ratios (the ratio of Sr to Ca amplifying by 1 000) along line transects from the core (0 μm) to the edge of sagittal otoliths of *Coilia nasus* collected from the Chikugo River Estuary.

Fig. 5. Strontium (Sr) imaging of *Coilia nasus* collected from the Chikugo River Estuary using otolith mapping analysis.
eggs, larvae) or even during their whole life history (like LJC03). Spawning of parent *C. nasus* in brackish water was possibly contributing to future population recruitment. Similar cases were found in some of those *C. nasus* from estuaries of several other rivers (e.g., the Changjiang River, Qiantangjiang River) along the coasts of the East China Sea (Dou et al., 2012; Jiang et al., 2014), indicating that they have the potential to develop into a non-anadromous stock in this region, as was the case of *C. nasus* found in the Changjiang Estuary by Dou et al. (2012).

In contrast, the results of both otolith life-history transect analyses (Table 3, Fig. 4) and X-ray intensity maps (Fig. 5) suggested that all fifteen specimens of *C. nasus* captured from the Chikugo River Estuary were of freshwater origin, unlike the aforementioned individuals of estuarine origin in *C. nasus* from the Rokkaku River. Nevertheless, the habitat use of Chikugo *C. nasus* was still more variable and complex than previously thought, considering their whole life history. All otoliths of the anchovies had a similar bluish color pattern and low Sr:Ca ratios (≤3) in their central regions, which were consistent with a presumed freshwater residency period (Yang et al., 2006; Jiang et al., 2014).

Adjacent to the bluish regions in the central patterns in Sr:Ca ratios along microprobe profiles varied among individuals. This variation in otolith Sr concentrations indicated that the anchovies from the same apparent spawning population in the Lojima section of the Chikugo River might have experienced a variety of movement patterns among fresh, brackish and sea waters in the period of life history after egg-larval stages, and, furthermore, even showed relatively more riverine-estuarine (e.g., ZHC01, ZHC02, ZHC03, ZHC04, ZHC14, ZHC15, ZHC17 and ZHC20), or estuarine-marine dependencies (ZHC05, ZHC06, ZHC07, ZHC09, ZHC10, ZHC12 and ZHC19), respectively.

Like the results of Yangtze *C. nasus* (Chen et al., 2017), the change in otolith Sr:Ca ratio and transition in concentric color rings (from blue to green, or further to red) corresponded to the salinity requirement of Rokkaku and Chikugo *C. nasus* during their migration from the spawning ground, from egg hatching to larval nursery, and to juvenile rearing habitats. It was unexpected to find that some *C. nasus* showed tendencies of freshwater or oligohaline requirement during their whole life histories. More specifically, the individuals of LJC08, LJC10, ZHC15 and ZHC20 almost consistently stayed in freshwater habitat except for entering brackish water for a very short time. This special strategy of habitat use implies that there may be unknown benefits (e.g., better feeding conditions) that can contribute substantially to the recruitment success and higher juvenile growth in endemic and restricted species like *C. nasus*, as Suzuki et al. (2014) indicated. Therefore, the current study suggests that *C. nasus* from both estuaries of the Rokkaku River and Chikugo River have a flexible capability of salinity habitat use with a high degree of migratory plasticity to decide whether to spend more or their life histories in freshwater, brackish, or seawater habitats. As previously emphasized by Kraus and Secor (2004) in their research on otolith microchemistry of white perch (*Morone americana*), we believe that a mosaic of habitats and developmental plasticity could be critical elements that have ecological significance, and both contingents play important roles in the population dynamics of *C. nasus*.

Many complex reasons are involved in the evolution and occurrence of migratory plasticity in diadromous fishes (Tsukamoto et al., 1998; Dou et al., 2012; Chen et al., 2017). Besides the natural ones, those from anthropogenic activity cannot be neglected. According to a previous study by Simanjuntak et al. (2015), a reclamation dike of the Ariake Bay has dramatically changed the environment and caused ecological problems associated with growth, breeding and systematics of *C. nasus*, including the occurrence of landlocked and non-anadromous *C. nasus* populations in the water areas inside the dike in the Ariake Bay.

Based on the results of microchemical analysis of the otolith Sr:Ca ratios and Sr-level map, the present study provides more objective and intuitive evidence and the most recent information on discriminating migratory patterns, and traces the habitat utilization patterns of *C. nasus* from estuaries of the Rokkaku River as well as the Chikugo River. This study reveals that either the former and/or the latter can play an important role as spawning and nursery grounds for *C. nasus*, and recognizes the availability of estuarine-origin individuals of this fish in the Rokkaku River Estuary, apart from the traditional riverine-origin individuals. The aforementioned results might be used for future effective management and artificial breeding of *C. nasus* in both Japan and China. Further studies need to be conducted for a better understanding of the spatial and temporal dynamics during the life history of *C. nasus*, in all river estuaries around the Ariake Sea, and the population connectivity of *C. nasus* between these rivers, the Chikugo River or the Rokkaku River.

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Response of winter cohort abundance of Japanese common squid *Todarodes pacificus* to the ENSO events

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Abstract

The Japanese common squid *Todarodes pacificus* is an economically important species with one year lifespan, which is significantly influenced by climatic and environmental variability. According to the fishery data of the winter cohort of *T. pacificus* from 2003 to 2012, as well as environmental data and the Oceanic Niño index (ONI), which was defined by the sea surface temperature (SST) anomaly in the Niño 3.4 region, variations in the SST, chlorophyll *a* (Chl *a*) concentration, suitable spawning area (SSA) and sea surface height anomaly (SSHA) on the spawning ground of *T. pacificus* were examined under the El Niño and La Niña conditions. Their influences on squid abundance (defined by catch per unit effort, CPUE) were further assessed. The results showed that seasonal changes were found in SST, Chl *a* and SSA on the spawning ground of *T. pacificus*. Correlation analysis suggested that annual CPUE was significantly positively correlated with Chl *a* and SSA (*p*<0.05), but had insignificant relationship with SST (*p*>0.05). Moreover, the El Niño and La Niña events tended to dominate the changes of SSA and Chl *a* concentration in the key area between 25°–29°N and 122.5°–130.5°E, driving the variability of squid abundance. However, this influence varied with the intensity of each anomalous climatic event: the weak El Niño event occurred, the spawning ground was occupied by waters with enlarged SSA but with extremely low Chl *a* concentration, leading to low squid recruitment, the CPUE then decreased; the moderate intensity of El Niño event resulted in shrunk SSA but with high Chl *a* concentration on the spawning ground, the squid recruitment and CPUE increased; the moderate intensity of La Niña events yielded elevated SSA and high Chl *a* concentration on the spawning ground, the squid recruitment and CPUE dramatically increased. Our findings suggested that the ENSO events played crucial effects on the incubating and feeding conditions of the winter cohort of *T. pacificus* during the spawning season and ultimately affected its abundance.

Key words: *Todarodes pacificus*, squid abundance, spawning ground, El Niño, La Niña


1 Introduction

It is well known that large-scale climate variability in the Pacific Ocean, particularly the El Niño-Southern Oscillation (ENSO), with its cycle including the warm (El Niño) and cold (La Niña) phases, significantly affects pelagic fish stocks (Lehodey et al., 2006). ENSO-driven environmental variability in the habitat coincides well with changes in distribution and abundance of many commercially important fish species, such as skipjack tuna *Katsuwonus pelamis* (Lehodey et al., 1997) and Pacific saury *Cololabis saira* (Tian et al., 2003). As to short-lived ommastrephid squid species, this species-environment relationship tends to be more sensitive (Anderson and Bodhouse, 2001). For example, recruitment conditions of the western winter-spring cohort of neon flying squid *Ommastrephes bartramii* as well as its fishing ground distributions were mediated by the El Niño and La Niña events. The sea surface temperature (SST) on the spawning ground during the El Niño years tended to be favorable for squid recruitment, and fishing ground would shift southward. However, the SST on the spawning ground appeared to be adverse to recruitment during the La Niña years, and the fishing ground shifted northward (Chen et al., 2007). In the Southeast Pacific Ocean, habitat quality and presence of jumbo flying squid *Dosidicus gigas* experienced dramatic fluctuations due to the anomalous environments. Suitable habitat areas of *D. gigas* were likely to expand under the La Niña conditions and shrink under the El Niño conditions (Yu et al., 2016).

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The Japanese common squid *Todarodes pacificus* is an ecologically important squid species extensively distributed throughout the temperate and warm waters between 20°N and 60°N in the Northwest Pacific Ocean (Murata, 1990). Due to its highly economic value, *T. pacificus* supports internationally commercial cephalopod fisheries in Japan, Korea and China, attracting large numbers of fishing vessels operating in the East China Sea (ECS), Sea of Japan and coastal waters along Japanese Islands in the Pacific side (Choi et al., 2008; Sakurai et al., 2013; Tang et al., 2015). According to spawning seasons, *T. pacificus* stock can be divided into summer, autumn and winter cohorts (Sakurai et al., 2000). The summer cohort of *T. pacificus* has a relatively small stock size. However, the winter cohort is the largest and most important one to make up the biomass of *T. pacificus* stock (Kawabata et al., 2006). In general, the distributions of spawning areas vary with seasons. The autumn cohort of *T. pacificus* mainly spawns in the Sea of Japan off southern Honshu Island and the Tsushima Strait between Korea and Japan. Eggs of this cohort are largely spawned between September and December (Goto, 2002; Yamamoto et al., 2002). For the winter cohort, spawning grounds occur in the East China Sea from the Kyushu Island south of Japan to Chinese Taiwan, with spawning peaking from January to April. Both cohorts migrate seasonally around the Japanese Islands from the southern spawning ground to the northern feeding ground (Hatanaka et al., 1985).

Previous studies have proved that climatic and oceanographic variations play far-reaching influences on *T. pacificus* stock (Rosa et al., 2011). The climate regime shift on decadal scales in the Northwest Pacific Ocean is considered as one of major drivers to determine the stock level of *T. pacificus*. Some studies have recognized that an inter-decadal recurring pattern of warm and cold regimes in the western Pacific perfectly corresponded to alternating high and low catches of *T. pacificus* in Korea and Japan (Sakurai et al., 2000). The importance of environmental conditions on the spawning ground, such as the size and spatial distribution pattern of suitable spawning area (SSA), is highly emphasized, which can be used to explain stock fluctuation of this squid (Sakurai et al., 2000; Rosa et al., 2011). However, currently, few studies have related this catch or stock size changes to interannual climate variability such as the ENSO phenomenon. How the *T. pacificus* stock responds to the El Niño and La Niña events has not yet been examined and discussed.

In this study, based on the fisheries data and three critical environmental factors including SST, SSA and chlorophyll a (Chl a) concentration, the relationship between the abundance of winter cohort of *T. pacificus* and the ENSO-related environmental variability has been examined. We hypothesize that variability in the abundance of the winter cohort of *T. pacificus* is primarily attributed to the change of its recruitment level during the early life stage. By analyzing the incubation and feeding conditions on the spawning ground under the El Niño and La Niña events with different intensity, we evaluate the potential effects of emerging event-to-event diversity of the ENSO conditions on the abundance of *T. pacificus* during 2003–2012. The purpose of this study is to elucidate response of abundance variation of *T. pacificus* to impacts of ENSO-mediated environmental variability on the spawning ground.

2 Materials and methods

2.1 Fishery and environmental data

The fishery data during 2003–2012 were obtained from the annually Japanese fisheries report for the winter cohort of *T. pacificus*. Data information included annual catches in Japan and Korea and the estimated catch per unit effort (CPUE) data. Fishing power in the study period was basically constant. Therefore, CPUE was regarded as a reliable index to indicate the squid abundance (Chen et al., 2008).

The environmental variables contained SST, Chl a concentration and sea surface height anomaly (SSHA) on the spawning ground (between 25°–35°N and 120°–135°E) of the winter cohort of *T. pacificus*. Data were obtained from remote sensing satellite databases. All the environmental data covered the main spawning months from January to April over 2003–2012. The monthly SST and SSHA data were acquired from the Live Access Server of National Oceanic and Atmospheric Administration (NOAA) OceanWatch dataset (http://oceanwatch.pfsc.noaa.gov/las/servlets/dataset). The spatial resolution of SST and SSHA was 0.1°×0.1° and 0.25°×0.25°, respectively. The monthly MODIS Chl a concentration data with spatial resolution of 0.05°×0.05° were sourced from the Asia-Pacific Data-Research Center (APDRC), University of Hawaii (http://apdrc.soest.hawaii.edu/data/data.php). All the environmental data were averaged on a 0.25°×0.25° latitude/longitude grid prior to analyses.

2.2 Climate index

The oceanic Niño index (ONI) becomes one standard measure for identifying the El Niño and La Niña events in the tropical Pacific (Li and Zhai, 2000; Tseng et al., 2012). In this study, the ONI was estimated by 5-month running mean of SST anomalies (SSTA) in the Niño 3.4 region (5°N–5°S, 120°–170°W). The ONI data from January to December during 2003–2012 were obtained from the following website: http://www.cpc.ncep.noaa.gov/products/analysis_monitoring/ensostuff/ensoyears2011.shtml. Based on a threshold of +/–0.5°C, the warm and cold periods of above and below normal SSTs were defined as the El Niño and La Niña events, respectively, when the threshold was met for a minimum of five consecutive overlapping months.

The intensity of El Niño or La Niña events were further categorized as weak, moderate, strong or very strong. The threshold was broken down into weak (with a 0.5 to 0.9 ONI, and with a −0.9 to −0.5 ONI), moderate (1.0 to 1.4, and −1.4 to −1.0), strong (1.5 to 1.9, and −1.9 to −1.5) and very strong (≥2.0, and ≤−2.0) events (http://ggweather.com/enso/oni.htm). For each intensity type, ONI must have equaled or exceeded the threshold for at least three consecutive overlapping three-month periods.

2.3 Evaluating variations in incubation and feeding conditions of *T. pacificus*

*Todarodes pacificus* dies immediately after breeding, its stock level, to a great extent, is determined by the recruitment (Sakurai et al., 2013). The incubation and feeding condition on the spawning ground during the early life stage are the main drivers to cause recruitment variability and consequently affect the abundance of *T. pacificus* (Sakurai et al., 1996; Yamamoto et al., 2007). Therefore, by examining the recruitment conditions under different ENSO periods, we could recognize the biophysical process how squid abundance responded to climate variability. In addition, in order to keep temporal and spatial consistency in this study, the spawning ground between 25°–35°N and 120°–135°E was chosen as the study area. Annual spawning month from January to April was regarded as our study period. The years with occurrence of anomalous climate events during spawning month were chosen to understand how the environmental conditions varied with the climate variability.
3 Results

3.1 ENSO episodes and squid abundance

Based on the definition of ENSO events, large fluctuations were observed in oceanic environments during 2003–2012 (Fig. 1). The Pacific Ocean experienced four El Niño events and three La Niña events during our study period. The former events occurred during January 2003–February 2003, July 2004–April 2005, September 2006–January 2007 and July 2009–April 2010, respectively. The latter events occurred in August 2007–June 2008, July 2010–April 2011 and August 2011–March 2012, respectively. Moreover, for each anomalous event, the intensity varied from year to year (Fig. 1). For example, the intensity of 2004/2005 and 2006/2007 El Niño events was categorized as weak intensity; the 2002/2003 and 2009/2010 El Niño events were categorized as moderate intensity; the 2011/2012 La Niña events were categorized as weak intensity; the 2007/2008 and 2010/2011 La Niña events were categorized as moderate intensity. No strong or very strong events were found during 2003–2012. Furthermore, spawning months from January to April in 2005, 2008, 2010 and 2011 corresponded to anomalous environmental events. The years of 2005 and 2010 were El Niño years, weak El Niño occurred in 2005, however, moderate El Niño occurred in 2010. Both 2008 and 2011 were moderate La Niña years. Thus, those four specific years were chosen in this study to examine how the recruitment conditions varied with the emerging event-to-event diversity of the ENSO conditions.

Annual CPUE of winter cohort of T. pacificus exhibited interannual variability over 2003–2012 with an average of 2.6×10^3 ind./(vessel·d) (Fig. 2). The CPUEs during 2007–2012 were higher than those during 2003–2006. The lowest CPUE occurred in 2006 with a value of 1.8×10^3 ind./(vessel·d); the highest CPUE reached up to 3.3×10^3 ind./(vessel·d) in 2007 and 2009. Additionally, the CPUE was 2.3×10^3 ind./(vessel·d), 2.5×10^3 ind./(vessel·d), 2.8×10^3 ind./(vessel·d) and 3.1×10^3 ind./(vessel·d), respectively, in 2005, 2010, 2008 and 2011. It was clearly found that the CPUEs were low in El Niño years (2005 and 2010) and high in La Niña years (2008 and 2011).

3.2 Seasonal environmental variations on the spawning ground

Environmental conditions on the spawning ground of T. pacificus showed apparently seasonal variations (Fig. 3). Comparing to other months, SST from January to April was relatively low ranging from 16.1°C to 17.9°C. The lowest SST occurred in February. After May, it gradually increased and reached a peak value of 28.6°C in August. The SST then decreased in the following months. Overall, the SST was high in summer and autumn, and low in spring and winter. Monthly Chl a concentration initially increased from January to May, followed by a decreasing trend between June and December. The Chl a concentration from March to June was much higher than that in other months, with a highest value of 1.83 mg/m^3 in April and May. The lowest Chl a concentration was 1.29 mg/m^3 in January. For SSA, it gradually decreased from January to June, high value concentrated in the spawning months. However, its value dramatically reduced and was approximately close to 0% during the summer season from July to September, and then increased after October. The highest SSA was 38.1% in January.

3.3 Relationship between CPUE and environmental variables

Correlation analysis was performed between CPUE and SST,
The results suggested that SST on the spawning ground was not significantly correlated with the CPUE for each month ($p>0.05$). Chl $\alpha$ concentration in January and April was significantly and positively correlated with the CPUE ($p<0.05$). Moreover, a significantly positive relationship was also found between CPUE and SSA in April ($p<0.05$). In addition, except the SST, annual average Chl $\alpha$ concentration from January to April as well as SSA was significantly positively correlated with the CPUE ($p<0.05$).

3.4 Variations in the environmental changes under different ENSO events

Figure 4 showed that SSA for winter cohort of $T.\text{pacificus}$ mainly occupied the waters from the northeast region of Chinese Taiwan to the southeast coast of Japan. Large SSA areas occurred along the eastern side of the Kuroshio Current in the western Pacific Ocean. Monthly SSA fluctuated in the four years, especially the SSA in 2010 obviously decreased. Furthermore, the sizes of SSA during 2005, 2010, 2008 and 2011 were quantified (Fig. 5). The SSA from January to April in 2005, 2008 and 2011 was larger than that in 2010. For details, the SSA in 2005 ranged from 34.2% to 37.1% with the highest value occurring in January and the lowest value occurring in March. The four-month average SSA in 2005 was 35.6%. In 2010, the SSA varied from 27.3% in April to 38.2% in January with an average value of 31.7%. In 2008, the SSA was between 31.7%–36.6%, the highest SSA occurred in January with the lowest value occurring in March, the average SSA was 34.4%. The SSA in 2011 ranged from 33.5% in February to 36.6% in April, the average SSA was 35.9%.

Obviously, the Chl $\alpha$ concentration in the coastal waters of China and Japan was higher than that in the offshore waters. Variations of the Chl $\alpha$ concentration in the near-shore regions tended to strongly fluctuate. Moreover, spatial and temporal changes of Chl $\alpha$ concentration were also found during the four years (Fig. 6). Figure 7 showed the monthly average Chl $\alpha$ concentration. The Chl $\alpha$ concentration was high in 2008, 2010 and 2011, and low in 2005. The Chl $\alpha$ concentration in 2005 ranged from 1.13 mg/m$^3$ in February to 1.45 mg/m$^3$ in April with an average of 1.32 mg/m$^3$. In 2010, the Chl $\alpha$ concentration varied from 1.32 mg/m$^3$ in February to 2.05 mg/m$^3$ in April with an average of 1.57 mg/m$^3$. The range of Chl $\alpha$ concentration in 2008 was between 1.39–1.79 mg/m$^3$, the highest value occurred in February with the lowest value occurring in April, four-month average

![Fig. 2. Annual CPUE of the winter-cohort of Japanese common squid $Todarodes$ $\text{pacificus}$ during 2003–2012.](image)

![Fig. 3. Monthly averaged SST, Chl $\alpha$ concentration and SSA on the spawning ground of the winter-cohort of Japanese common squid $Todarodes$ $\text{pacificus}$ during 2003–2012.](image)
value was 1.57 mg/m$^3$. For the Chl $a$ concentration in 2011, it ranged from 1.31 mg/m$^3$ in February to 2.03 mg/m$^3$ in April with an average of 1.56 mg/m$^3$.

In fact, the Chl $a$ concentration spatially consistent with the SSA area in Fig. 4 was very low (<1.0 mg/m$^3$) comparing to other regions on the spawning ground. Variability of the Chl $a$ concentration in these areas was difficult to differ in the four years. On the other hand, small difference was found in the four years by using the four-month average Chl $a$ concentration on the spawning ground. Therefore, it was biased using the average Chl $a$ concentration on the whole spawning ground to examine the feeding environment for $T.\ pacificus$. To address this question, we examined the spatial distribution of the correlation coefficients between Chl $a$ concentration on the spawning ground and the CPUE of $T.\ pacificus$ during January–April in the period of 2003–2012. A region with extremely high positive correlation coefficients occurred in the waters between 25°–29°N and 122.5°–130.5°E (Fig. 8). The location of this area was in consistence with the spatial distribution of the SSA. Thus, we considered the feeding environment in this area was a key factor that strongly influenced the CPUE of $T.\ pacificus$.

The Chl $a$ concentration in the key area between 25°–29°N and 122.5°–130.5°E was examined in these four years (Fig. 9). High and low Chl $a$ concentration occupied the northwestern and southeastern waters of this area, respectively. Comparing with the year of 2010, monthly Chl $a$ concentration during January to April in 2005 was low. The Chl $a$ concentration in 2008 and 2011 tended to be enhanced for each month. By quantitative analysis, the results indicated that the Chl $a$ concentration in 2005 ranged from 0.28 mg/m$^3$ in February to 0.42 mg/m$^3$ in April with an average of 0.35 mg/m$^3$. The Chl $a$ concentration in 2010 ranged from 0.33 mg/m$^3$ in January to 0.71 mg/m$^3$ in April with an average of 0.47 mg/m$^3$. In 2008, the Chl $a$ concentration varied from 0.27 mg/m$^3$ in January to 0.65 mg/m$^3$ in March with an

<table>
<thead>
<tr>
<th>Month</th>
<th>SST</th>
<th>Chl $a$</th>
<th>SSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jan.</td>
<td>-0.018</td>
<td>0.612 (p&lt;0.05)</td>
<td>0.182</td>
</tr>
<tr>
<td>Feb.</td>
<td>0.417</td>
<td>0.211</td>
<td>0.502</td>
</tr>
<tr>
<td>Mar.</td>
<td>0.361</td>
<td>0.148</td>
<td>0.309</td>
</tr>
<tr>
<td>Apr.</td>
<td>0.177</td>
<td>0.710 (p&lt;0.05)</td>
<td>0.631 (p&lt;0.05)</td>
</tr>
<tr>
<td>4-month average</td>
<td>0.280</td>
<td>0.694 (p&lt;0.05)</td>
<td>0.637 (p&lt;0.05)</td>
</tr>
</tbody>
</table>

Table 1. Correlation between the environmental variables including SST, SSA and Chl $a$ concentration and the CPUE of Japanese common squid $Todarodes\ pacificus$ during 2003–2012

Fig. 4. Spatial distribution of SSA on the spawning ground of the winter-cohort of Japanese common squid $Todarodes\ pacificus$ from January to April in the El Niño years of 2005/2010 and in the La Niña years of 2008/2011, respectively.
average of 0.45 mg/m³. The Chl \(a\) concentration in 2011 fluctuated from 0.48 mg/m³ in February to 0.86 mg/m³ in April with an average of 0.60 mg/m³.

Variability of SSHA during January to April in 2005, 2010, 2008 and 2011 was showed in Fig. 11. Except in March, highly positive SSHA tended to occupy the regions between 25°–29°N and 122.5°–130.5°E and eastern waters of 131°E in January, February and April in 2005, indicating that the SSA areas were covered by highly elevated SSHA on the spawning ground in this year. On the contrary, large portion of waters with negative SSHA oc-

**Fig. 5.** Monthly SSA on the spawning ground of the winter-cohort of Japanese common squid *Todarodes pacificus* from January to April in the El Niño years of 2005/2010 and in the La Niña years of 2008/2011, respectively. The red line indicated the average SSA from January to April in each year.

**Fig. 6.** Spatial distribution of Chl \(a\) concentration on the spawning ground of the winter-cohort of Japanese common squid *Todarodes pacificus* from January to April in the El Niño years of 2005/2010 and in the La Niña years of 2008/2011, respectively.
The scientific issue that exploring the underlying causes of the variability in abundance (Roberts, 2005; Yu et al., 2013). Present studies suggested that recruitment variability during the early life stage for squids was highly vulnerable to the biological and physical environment (e.g., incubation and feeding conditions) on the spawning ground (Nevárez-Martínez et al., 2006; Chen et al., 2007). Therefore, it necessitated the scientific issue that exploring the underlying causes of the links between the ENSO events, the dominant climate variability in the Pacific Ocean, and stock dynamics of T. pacificus. Such relationship often had good predictive capability for its abundance in a short term. However, it was poorly understood so far for T. pacificus. Hence, this study connected the ENSO-mediated variability in incubation and feeding conditions to CPUE in order to clarify the influences of climate variability on abundance of T. pacificus.

Due to profound influence of SST and Chl $a$ density on temperature-depended survival rates, habitat suitability and prey density for squid paralarvae and juveniles, many efforts examined these two environmental factors on squid spawning ground to evaluate the potential impacts on fluctuations of squid abundance and catch (Waluda and Rodhouse, 2006). For example, Cao et al. (2009) related the abundance of O. bartramii during 1995–2004 with the SSA on the spawning ground and preferred habitat areas (PHA) on the feeding ground. They inferred that SSA had limited impacts on the recruitment between the two years. However, high SST waters falling within enhanced Chl $a$ concentration on the spawning ground in 1999 might cause increased recruitment and squid abundance. Whereas low SST occurred on the spawning ground matching with reduced Chl $a$ concentration resulted in decreased recruitment and squid abundance.

Our findings suggested that SST, Chl $a$ concentration and SSA had substantial changes on a seasonal time scale. Relative low SST, high Chl $a$ concentration and enlarged SSA during January to April could favor the adaptation of T. pacificus to their environment. Spawning in these months appeared to be in accord with the reproductive strategy (Rocha et al., 2001). Moreover, the results from the correlation analysis (Table 1) in this study indicated that variability in squid abundance was closely associated with the SSA and Chl $a$ concentration on the whole spawning months from January to April. Particularly, January and April for Chl $a$ and April for SSA tended to be the most important period influencing squid abundance of T. pacificus.

There were some studies that evaluate the relationship between the SSA and stock level of T. pacificus, but conclusions varied with different research. Sakurai et al. (2002) suggested that stock fluctuation of winter cohort of T. pacificus was related to...
winter-spawning area. A cool regime would shrink winter-spawning area in the East China Sea and decrease adult stock. While a warm regime would shift the distribution of winter-spawning ground in the Sea of Japan overlapping with autumn-spawning area in the East China Sea, such spatial distribution of spawning ground increased the stock size. However, for the autumn cohort, no significant changes of SSA on the spawning ground were reported between different climate regimes. Besides, Rosa et al. (2011) proposed that stock level of *T. pacificus* was determined by distribution patterns of spawning areas, not by the size of SSA. The discontinuity of spawning-ground distribution was likely to reduce the catch in the following fishing season. Comparing to their studies, our study used data sources with different study period, and the definition of SSA was only based on one factor (i.e., SST), which might cause the differences in these findings.

**Fig. 9.** Spatial distribution of Chl *a* concentration between 25°–29°N and 122.5°–130.5°E from January to April in the El Niño years of 2005/2010 and in the La Niña years of 2008/2011, respectively.

**Fig. 10.** Monthly Chl *a* concentration between 25°–29°N and 122.5°–130.5°E from January to April in the El Niño years of 2005/2010 and in the La Niña years of 2008/2011, respectively. The red line indicated the average Chl *a* concentration between 25°–29°N and 122.5°–130.5°E from January to April in each year.
The SSTA in the Niño 3.4 region was successfully used to define the ENSO events and related to the marine pelagic fish species (Arcos et al., 2001; Sun et al., 2006; Han et al., 2009). For example, the gravity centers of fishing ground of fish species such as *D. gigas* (Xu et al., 2011) and *K. pelamis* (Wang and Chen, 2013) were strongly regulated by the El Niño and La Niña events which were defined by the Niño 3.4 SSTA. Thus, we utilized the SSTA in the Niño 3.4 region to define the ENSO events in this study. Our studies suggested that variability in squid abundance of *T. pacificus* was primarily driven by the SSA and Chl *a* concentration in the key area between 25°–29°N and 122.5°–130.5°E, which were mediated by the El Niño and La Niña events. However, the influence of each anomalous climate event on squid abundance varied with its intensity. The weak El Niño event might yield enlarged SSA and extremely low Chl *a* concentration on the spawning ground, leading to low squid recruitment, the CPUE of *T. pacificus* decreased. The moderate intensity of El Niño event might result in shrunk SSA but with high Chl *a* concentration on the spawning ground, the squid recruitment and CPUE increased. The moderate intensity of La Niña events might yield both elevated SSA and enhanced Chl *a* concentration on the spawning ground, the squid recruitment and CPUE dramatically increased. Our findings presented recruitment variability of the winter cohort of *T. pacificus* in response to the El Niño and La Niña event years from 2005 to 2011.

![Fig. 11. Spatial distribution of SSHA on the spawning ground of the winter-cohort of Japanese common squid *Todarodes pacificus* from January to April in the El Niño years of 2005/2010 and in the La Niña years of 2008/2011, respectively.](image-url)

**Table 2.** Regression model between the environmental variables (suitable spawning area, SSA and Chl *a* concentration) and the CPUE of Japanese common squid *Todarodes pacificus*

<table>
<thead>
<tr>
<th>Model</th>
<th>95% CI</th>
<th><em>p</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>CPUE</td>
<td>$a_0=-5.158$</td>
<td>$-10.048$ to $-0.267$</td>
</tr>
<tr>
<td></td>
<td>$a_1=0.196$</td>
<td>0.065 to 0.328</td>
</tr>
<tr>
<td></td>
<td>$a_2=1.669$</td>
<td>0.106 to 3.231</td>
</tr>
<tr>
<td>$P_{1,n}$</td>
<td>average SSA from January to April in year <em>n</em></td>
<td></td>
</tr>
<tr>
<td>$P_{2,n}$</td>
<td>average Chl <em>a</em> concentration between 25°–29°N and 122.5°–130.5°E from January to April in year <em>n</em></td>
<td></td>
</tr>
</tbody>
</table>

Correlation coefficient $r=0.830$; $R^2=0.689$; $F=7.762$; $p=0.017$
Niño and La Niña events with different intensity. Alabia et al. (2016) demonstrated the potential habitat of the autumn cohort of O. b. bartramii in response to ENSO flavors, they found the East Pacific (EP)-El Niño created better feeding environments and yielded larger suitable habitat while the Central Pacific (CP)-El Niño reduced the suitable habitat. Due to the El Niño and La Niña events showed difference in intensity, time duration and spatial distribution, response of squid stocks to ENSO events should be analyzed case by case. In fact, in our study, the anomalous climate event in 2005 was the EP-El Niño, whereas the anomalous climate event in 2010 was the CP-El Niño. Comparing to the EP-El Niño in 2005, the CP-El Niño in 2010 was stronger in intensity and longer in duration, leading to shrink SSA and enhanced Chl a concentration in the study region, it facilitated the environmental effects and increased squid abundance of T. pacificus.

Though the SSA was low in 2010 and the Chl a concentration was high, the CPUE was still at a high level, implying that the feeding condition might be more important than the size of suitable spawning areas to the squid recruitment. This explained the reason why the CPUE in 2010 was higher than that in 2005. More so, the feeding environment bounded by 25°–29°N and 122.5°–130.5°E had important implications for the winter cohort of T. pacificus and was possibly affected by the SSA on the spawning ground. We inferred the possible biophysical process through which the dynamic of SSA influenced the Chl a concentration and further the squid abundance under different ENSO events (Figs 9, 10 and 11): in contrast to the weak EP-El Niño year in 2005, a large portion of negative SSAs, which was indicative of strengthened upwelling (Yu et al., 2016), widely occurred on the spawning ground of T. pacificus in the moderate EP-El Niño year of 2010 and the moderate La Niña years of 2008 and 2011. The nutrient-rich bottom waters were transported to the surface spawning ground, this process led to extension of high Chl a in the key areas between 25°–29°N and 122.5°–130.5°E, thereby enhancing the primary productivity and prey availability and consequently increasing the abundance of T. pacificus.

As shown in Table 2, the regression model successfully captured the positive relationship between the CPUE of T. pacificus and the SSA and Chl a concentration in the key areas were between 25°–29°N and 122.5°–130.5°E. However, inevitably, there were some limitations in this study. For instance, as current (Kim et al., 2015) and salinity (Furukawa and Sakurai, 2008) shown importance in regulating the T. pacificus stocks, our studies only included SST and Chl a concentration. Besides, we only used the SST to define the SSA, which might introduce biases in the analysis. Comparing to the years with anomalous environments, the CPUE in the normal years also fluctuated, such as the lowest CPUE in 2006 and highest CPUE in 2007. In order to perform a comprehensive evaluation of impacts of different climatic events on T. pacificus stocks, future studies should involve with more environmental variables and extend the study period including the consideration of the normal climate condition.

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Spatial pattern of macrobenthic communities along a shelf-slope-basin transect across the Bering Sea

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Abstract
Due to its unique geological location, the Bering Sea is an ideal place to investigate the water exchange and ecosystem connectivity of the Pacific Ocean–Arctic Ocean and subarctic–Arctic region. Based on a number of summer surveys (July to September, 2010, 2012 and 2014), macrobenthic communities and their spatial-temporal patterns are exhibited for the majority of the Bering Sea (53°59′–64°36′N). The results show that the macrobenthic communities were dominated by northern cold-water species and immigrant eurythermic species, and the communities assumed a dispersed and patchy distribution pattern. Polychaetes (Scoloplos armiger), crustaceans (Ceradocus capensis) and sea urchins (Echinarchnium parnum) were the major dominant groups in the shallow shelves; the sea star (Ctenodiscus crispatus) and the brittle star (Ophiura sarsi) were the main dominant groups in the continental slope; whereas small polychaetes (Prionospio malmgenri) dominated the basin area. Sediment type, water depth, and currents were the major factors affecting the structure and spatial distribution of the macrobenthic communities. Compared with other seas, the shallow areas of the Bering Sea showed an extremely high-standing biomass. In particular, the northern shelf area (north of St. Lawrence Islands and west of 170°W), which is primarily controlled by Anadyr Water, is an undersea oasis. In contrast, a deficiency in the downward transport of particulate organic carbon has resulted in a desert-like seabed in the basin area. By comparing our results to previous studies, we found that macrobenthic communities of the Bering Sea have undergone significant structural changes in recent decades, resulting in a decrease in abundance and an increase in biomass. In addition, populations of amphipods and bivalves in the northern shelves have decreased significantly and have been gradually replaced by other species. These changes might be associated with advanced seasonal ice melting, changes in organic carbon input, and global warming, indicating that large-scale ecosystem changes have been occurring in the Bering Sea.

Key words: macrobenthos, community structure, Arctic, Bering Sea, environmental drivers


1 Introduction
The Bering Sea is the only link between the Arctic Ocean and the Pacific Ocean. Due to its unique geographical location, this sea area is an ideal place to investigate the water exchange and ecosystem connectivity of the Pacific Ocean–Arctic Ocean and subarctic–Arctic region (Clement et al., 2005; Woodgate and Aagaard, 2005). Although located at a high latitude with 7–8 months of seasonal snow cover, the Bering Sea remains one of the world’s most productive regions (Berger et al., 1987; Grebmeier et al., 1988; Piepenburg, 2005; Wang et al., 2014a; Lin et al., 2016). The Bering Sea is rich in fishery resources, especially in fish and crabs, which support a plurality of higher predators such as cetaceans, seals, elephant seals, and sea birds that migrate to the area for feeding (Springer et al., 1996; Moore et al., 2003; Lovvorn et al., 2003; Ray et al., 2006; Dehn et al., 2007). In the continental slope region at the junction of the basin and the shelf of the Bering Sea, the Bering Sea Green Belt has a primary productivity and a seabed habitat secondary productivity (calculated by carbon) of 175–275 g/(m²·a) (Okkonen et al., 2004) and (4.2±1.8) g/(m²·a) (Lin et al., 2016), respectively.

Macrobenthos play an important role in polar marine ecosystems. Because these organisms have a slow growth rate and a long life cycle, they are resistant to interannual change and small fluctuations in water column productivity (Carey, 1991). Thus, macrobenthos are good indicators of potential changes in ecosystems. Changes in the macrobenthic communities at a time-
cale of years to decades can predict long-term changes in ecosystems caused by climate effects (Dunton et al., 2005). The retreat of sea ice and increased glacial melt caused by global warming have seriously affected biodiversity and the community structure in the Bering Sea (Wlodarska-Kowalczuk and Wereslaski, 2001; ACIA, 2004; Perovich et al., 2015). In the last 25 years, the biomass of the benthic Greenland halibut (Greenland turbot) has decreased by more than 80%, whereas that of pelagic fishes, such as cod, has increased by approximately 400%. An abrupt change in snow crab biomass is believed to be closely associated with the sea ice retreat caused by rising temperatures (Siddon and Zador, 2017). Human activities are also a major cause of interference in the region; both business development and overfishing have led to sharp changes in the macrobenthic population and community structure of the Bering Sea and surrounding waters. For example, marine mammals on the top of the food chain (such as sea lions) decreased by more than 50% between the 1950s and the 1980s, and the population of harbor seals decreased by 90% since the 1970s (Pitcher, 1990).

The shallow shelves and slope of the Bering Sea are critically influenced by water column production, organic carbon cycling, and pelagic-benthic coupling. Short food chains and shallow depths are characteristic of high-productivity areas in this region, and thus, changes in lower trophic levels can rapidly impact higher trophic organisms, including pelagic- and benthic-feeding marine mammals and seabirds (Gremerer, 2006a).

The latest research shows that the amplified Arctic warming over the past decade has significantly contributed to a continual global warming trend (Huang et al., 2017), hence there is a critical need for biological, geological, and chemical comprehensive analysis and time series comparison to observe and evaluate the changing Arctic ecosystem. Using data acquired in the Bering Sea during the 4th, 5th, and 6th Chinese National Arctic Research Expeditions (CHINAREs) combined with previous survey data and literature, this study aims to answer the following questions: (1) What is the spatial variability of macrobenthic communities in the Bering Sea along the shelf-slope-basin transect? (2) What is the change in benthic macrobenthic communities of the Bering Sea in the context of climate change? (3) Is there a quantifiable relationship between the spatial differences in macrobenthic communities and environmental factors? Answering these questions will allow us to investigate the dynamics of the macrobenthic communities of the subarctic seas and its response to environmental change. Our quantitative data on macrobenthos will improve our understanding of benthic production processes and ecosystem dynamics in the context of rapid climate change.

2 Methods

2.1 Survey area

The Bering Sea is the northernmost marginal sea on the coast of the Pacific Ocean, and connects the Arctic Ocean in the north through the Bering Strait to the Pacific Ocean in the south across the Aleutian Islands. The Bering Sea has a complex seabed terrain with extensive variation. Deep-sea basins are found in the south and west, and large shelf areas exist in the north and east. The key currents of this region were the more saline, nutrient-rich Anadyr Water (AW) transiting northward on the western side, and fresher, more nutrient-limited Alaska Coastal Water (ACW) flowing northward on the eastern side, and intermediate salinity, nutrient-rich Bering Shelf Water (BSW) lies between AW and ACW. Much of the northern Bering Shelf is ice-covered in winters but ice-free in summers, and the growth of ice over southern deep water is limited by warm water in the central basin.

2.2 Sampling methodology

Macrobenthic samples were collected at 46 stations in the Bering Sea (Fig. 1) while aboard the R/V Xuelong during the 4th CHINARE (July to September 2010), 5th CHINARE (July to September 2012), and 6th CHINARE (July to September 2014). Thereinto, 33 stations at depths ranging from 19 m to 105 m were located on the Bering Shelf, which were divided into three sectors comprising the northern (NB), eastern (EB) and western (WB) Bering Shelf; seven stations were located on the Bering Slope (BS) at depths ranging from 119 m to 258 m; six stations were located on the Bering Basin (BB) at depths ranging from 2,603 m to 3,873 m. To facilitate discussion, abbreviations for specific regions were adopted.

One sample was collected at each station using a 0.25 m² box corer (50 cm × 50 cm × 60 cm). Each sample was rinsed through 0.5-mm mesh sieves, and the residue containing the macrobenthos was fixed in 7% formaldehyde for processing. Macrobenthos were identified to the lowest possible taxonomic level, and individuals within each taxon were counted and weighed. Taxon names were cross checked against the World Register of Marine Species (http://marinespecies.org/).

Environmental variables, including bottom temperature and salinity at the base of the water column, were quantified at each station using a Sea-Bird Electronics (SBE911 Plus) CTD system. Grain analysis (percentage of sand, silt and clay) of the filtered sub-samples was performed at each station using a Malvern Mastersizer laser particle sizer according to Yao et al. (2014). The median particle diameter (Md) and sorting values were also calculated. The total organic carbon (TOC) and total nitrogen (TN) contents in the sediments were measured with a CHN analyzer (Vario EL III) after the samples were freeze-dried according to Qiao et al. (2011). The environmental factors measured are summarized in Table 1.

2.3 Data treatment and analysis

PRIMER 6.0 was used to calculate the Bray-Curtis similarity coefficients, on which the similarity matrix and the cluster analysis were constructed. To mitigate the effects of rare and opportunistic species on the macrobenthic structure analysis, a pre-treatment was performed on the raw data to exclude species with a relative abundance of less than 0.5%. Species with a relative abundance greater than 3% at any given station were retained. To balance the roles of the dominant and rare species in the macrobenthic structure, the abundance data were subjected to fourth root conversion and standardization. To identify the characteristic species for each of the macrobenthic groups, we used similarity profile (SIMPROF) to test different groups based on the similarity percentage (SIMPER) to sort the inter-group percentages of the average similarity contribution.

We analyzed the relationship between the environmental variability and the macrobenthic communities using direct gradient analysis. The unimodal ordination method of canonical correspondence analysis (CCA) was chosen based on a preliminary detrended correspondence analysis on macrobenthic communities, which indicated that the longest length of gradient (20.269 3) was longer than 4, suggesting that the majority of taxa exhibited a linear response to environmental variation (Leps and Šmilauer, 2003). The collinearity between environmental factors and macrobenthic communities was tested to prevent over-interpretation.
(Blanchet et al., 2008; Borcard et al., 2011), followed by a Monte Carlo permutation test (999 permutations under reduced model). The results showed that the correlation between environmental factors and macrobenthic communities was reliable (Pseudo-F=1.230 984, Significance=0.004). The above analysis was performed using R 3.4.2 and the vegan library (Oksanen et al., 2017).

3 Results

3.1 Species composition and distribution

A total of 12 phyla and 239 species of macrobenthos were identified from the obtained samples, of which polychaetes were the most abundant (with a total of 110 species), whereas crustaceans, mollusks and echinoderms represented 49, 44 and 12 species, respectively (Fig. 2). The dominant species in the shelves of the Bering Sea included Scoloplos armiger, Ceradocus capensis, and Echinarchnus parma; those in the slope area included Ctenodiscus crispatus, Ophiura sarsi, and Eudorella pacifica; and those in the basin area included Prionospio malmgreni, Dasybranchethus fauveli, and Ampelisca brevicornis.

3.2 Distribution of abundance and biomass

The average abundance of macrobenthos of all the stations was $(727\pm320)$ ind./m$^2$, and the average densities of the five sectors were as follows: NB ($(1\ 027\pm156)$ ind./m$^2$) > EB ($(926\pm1\ 001)$ ind./m$^2$) > WB ($(700\pm602)$ ind./m$^2$) > BS ($(572\pm752)$ ind./m$^2$) > BB ($(28\pm29)$ ind./m$^2$). The average abundance of the NB was characterized by crustaceans, whereas polychaetes were more abundant in the other areas (Fig. 3).

The mean biomass was $(597.2\pm1\ 603.6)$ g/m$^2$, and the average biomass of the five sectors were as follows: NB ($(1\ 435.4\pm2\ 762.6)$ g/m$^2$) > WB ($(357.8\pm343.8)$ g/m$^2$) > BS ($(332.6\pm223.1)$ g/m$^2$) > EB ($(107.6\pm108.5)$ g/m$^2$) > BB ($(0.4\pm0.4)$ g/m$^2$). The average biomasses of the NB and BS were characterized by echinoderms, whereas the EB and WB were characterized by mollusks. The average biomass of the BB was characterized by polychaetes (Fig. 4).

3.3 Community structure

The macrobenthic data collected from the 44 stations (two of which showed no presence of organisms) were subjected to cluster analysis (Fig. 5). The macrobenthic communities were divided into 16 groups with a 16% similarity threshold. The compositions were significantly different among the groups ($R=0.847$, $p=0.001$). Using SIMPER, species in each group (excluding the groups that only had one station) that had an accumulated contribution rate of 80% (Table 2) and had a significant inter-group contribution rate were used as characteristic species to describe the macrobenthic communities. The geographical distributions of the groups are shown in Fig. 6.
3.4 Relationship between macrobenthos and environmental factors

The total interpretation of CCA for the macrobenthos was 24.6% (Fig. 7). Among the environmental variables, Md ($r^2=0.7597$, $p=0.001$), sand% ($r^2=0.3071$, $p=0.002$), silt% ($r^2=0.4284$, $p=0.008$), clay% ($r^2=0.3407$, $p=0.032$), TOC ($r^2=0.5057$, $p=0.002$), and TN ($r^2=0.5240$, $p=0.001$) were significantly related to the community structure. For example, Group 2 was significantly positively correlated with sand%, and Groups 9 and 10 were significantly positively related to clay% and TOC.

4 Discussion

The results showed that the macrobenthic communities in the Bering Sea were characterized by northern cold-water species and immigrant eurythermic species. Most of the species were distributed in a wide range, whereas the indigenous species were rare. For example, the large crab *Chionoecetes opilio* is widely distributed in the Arctic Ocean, Barents Sea, northern Atlantic Ocean and St. Lawrence Bay. The small amphipod *Ceradocus capensis* was found in the South China Sea. The macro echinoderm *Echinarchnus parma* was found in the Arctic.
Ocean, the North Atlantic Ocean, and the waters near Canada. *Ctenodiscus crispatus*, a characteristic species of continental slope, is widely distributed in the Sea of Japan, the northern Atlantic Ocean, the Okhotsk Sea, the Barents Sea, and the waters around Canada and the United States. *Ophiura sarsii*, which is one of the major characteristic species of the Bering Sea and the Arctic Ocean, has a distribution range extending to the northern Atlantic Ocean, the northern Pacific Ocean, the Sea of Japan, the St. Lawrence Bay, the Gulf of Mexico, the Yellow Sea, and the waters around Europe. Moreover, polychaetes such as *Scoloplos armiger* and *Heteromastus filiformis* are distributed in the Arctic Ocean, the northern Atlantic Ocean, the northern Pacific Ocean, the Mediterranean Sea, the North China Sea, and the East and South China Seas. Mollusks such as *Nuculana pernula* and *Megayoldia thraciaeformis* are distributed in the North Atlantic, the Barents Sea, and the waters around Canada (GBIF Secretariat, 2017; WoRMS, 2017; Huang and Lin, 2012a, b).

Similar to the macrobenthos of the western Arctic Ocean, but significantly different from those in the Southern Ocean (Brey and Gerdes, 1998; Wang et al., 2014b; Lin et al., 2016), the macrobenthic communities in the Bering Sea were not a typical local community but contained diverse community structures derived from spatial heterogeneity. The Bering Sea is a marginal sea of the Pacific Ocean and is located in the subarctic region. Nutrient-rich warm waters of the Pacific Ocean pass the Bering Strait and enter the Arctic Circle, allowing species exchange between the Bering Sea and other northern seas (Piepenburg, 2005). The macrobenthic communities in the Bering Sea were exhibited a dispersed and patchy distribution pattern.

The *Chionoecetes opilio*-*Echinarachnius parma* group (Group 2) was primarily distributed in the sandy NB (with a water depth of approximately 40 m) and exhibited a simple composition. The large animals *Chionoecetes opilio* and *Echinarachnius parma* were the major characteristic species and had average densities of 13 ind./m$^2$ and 156 ind./m$^2$, respectively, and average biomasses of 257.1 g/m$^2$ and 3 660.5 g/m$^2$, respectively. The *Ctenodiscus crispatus*-*Cyclocardia crebricostata*-*Megayoldia thraciaeformis* group (Group 7) was mainly distributed in the BS with sandy silt, was found at a water depth of 119–130 m, and displayed a simple community structure. *Ctenodiscus crispatus* was the characteristic species and had an average abundance and biomass of 16 ind./m$^2$ and 255.6 g/m$^2$, respectively. The *Nuculana pernula*-Tharyx sp.-*Ophiura* sp. group (Group 9) was primarily distributed in the WB (32–102 m water depth) in sandy silt sediment. *Nuculana pernula* was the characteristic species, with an average abundance and biomass of 30 ind./m$^2$ and 23.9
g/m², respectively. The *Heteromastus filiformis-Praxillella praetermissa-Nephtys ciliata-Ophiura sarsii* group (Group 10) was rich in species, complex in structure, and wide in distribution. *Heteromastus filiformis* and *Ophiura sarsii* were the characteristic species and were observed in the WB and BS at 35–258 m in silty sand or sandy silt sediment. The *Scoloplos armiger-Glycinde wireni-Sternaspis scutata* group (Group 11) was simple in structure. The polychaete *Scoloplos armiger* was the characteristic species and was distributed in the EB and WB at a depth of 32–49 m in a sandy or silty sediment. The *Ceradocus capensis-Scoloplos armiger-Nephtys caeca-Byblis gaimardii* group (Group 12) was characterized by small amphipods and polychaetes, was distributed in the NB and EB, and was found at a water depth of 19–40 m in sandy sediment.

The environmental factors affecting macrobenthic communities are complex and diverse (Robertson, 1979; Cusson and Bourget, 2005; Grebmeier et al., 2006a; Bolam and Eggleton, 2014; Lin et al., 2016). Blanchard et al. (2013a, b) found that the sharp change in the water depth from the shelves to the basin and the differences in sediment conditions and organic matter supply were the main factors resulting in differences in the macrobenthic distribution. Grebmeier et al. (2006a) argued that the nutrient-rich warm waters of the Pacific Ocean and the violent pelagic-benthic coupling were the major factors influencing the macrobenthic distribution in the Arctic regions. Additionally, the impact of the pelagic-benthic coupling on the macrobenthos of the Arctic was much greater than that on the macrobenthos of temperate and tropical waters (Petersen and Curtis, 1980). Comprehensive analysis revealed that, among the data obtained by simultaneous observation, the sediment type, water depth and sedimentary organic matter content were the major factors affecting the community structure and spatial distribution of the communities.

![Fig. 4. Spatial distribution of macrobenthos biomass in the Bering Sea.](image-url)

![Fig. 5. Bray-Curtis similarity cluster analysis of macrobenthic communities in the Bering Sea.](image-url)
The sediment type is an important factor that affects the natural distribution of macrobenthos, and is the result of the long-term natural selection of the macrobenthos (Hopcroft et al., 2008; Bolam et al., 2010). The sediment of the shelf and slope areas of the Bering Sea is dominated by silty sand/sandy silt, whereas the basin area is characterized by clayey silt. The diversity and standing biomass of the macrobenthos from areas with coarser sediment were higher than those from areas with muddy habitats.

Water depth and currents determine the downward transport of available particulate organic carbon (POC) from the upper waters for macrobenthos, as well as the pelagic-benthic coupling strength. The NB is at the intersection of the Alaska Coastal Water (ACW), Anadyr Water (AW), and Bering Sea Slope Water.

### Table 2. Simper similarity analysis based on the macrobenthic communities composition of the Bering Sea

<table>
<thead>
<tr>
<th>Group</th>
<th>Total similarity</th>
<th>Species</th>
<th>Average abundance</th>
<th>Average similarity</th>
<th>Contribution rate/%</th>
<th>Cumulative percentage/%</th>
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<td>36.12</td>
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<td>Meganyoldia thracaiformis</td>
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<td>42.36</td>
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<td>90.5</td>
</tr>
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</table>
Due to tight pelagic-benthic coupling and the high primary and secondary productivity of the water column (Grebmeier et al., 1988), a high-standing biomass is present in this area, compared with other seas (e.g., the Barents Sea, the UK continental shelf, the English Channel, the North Sea, the Weddell and Lazarev Sea, Porsangerfjord of Northern Norway, the Beaufort Sea, and the Arctic Ocean) (Brey and Gerdes, 1998; Cooper et al., 2008; Bolam et al., 2010; Kędra et al., 2013; Fuhrmann et al., 2015; Lin et al., 2016). In the Asian continent facing side of the northern Bering Sea (west of 170°W, north of St. Lawrence Island), where the nutrient-rich AW and a strong upwelling present, the average abundance and biomass of macrobenthos reach (1 505±3 062) ind./m² and (2 774.3±3 513.9) g/m², respectively, which can be called a “Bering benthic oasis”. However, on the side facing Alaska (east of 170°W), where the nutrient-limited ACW dominant, the average abundance and biomass of macrobenthos are much lower, at (551±405) ind./m² and (96.5±75.7) g/m², respectively. Unlike the slope area of the Chukchi Sea, the Bering Slope is another oasis with high water column production, which
forms the “Bering Sea Green Belt” and has a primary production between 175 and 275 g/(m²·a). Tidal mixing at the shelf-break front and transverse circulation in the Bering Slope Current, which includes eddies near the shelf edge, result in vertical transport of pelagic nutrients to the seafloor (Springer et al., 1996). Rich food sources have resulted in high-standing benthic biomass in this area. The deep Bering Basin, which accounts for nearly half of the Bering Sea, exhibits typical high-nutrient, low-chlorophyll (HNLC) properties (Banse and English, 1999). The water depth has become an inhibitor for the transfer of POC in water column to seafloor. The deficiency in food sources has resulted in a desert-like seabed.

Previous studies have shown that the average abundance and biomass of macrobenthos were 4,752 ind./m² and 300–400 g/m² (Grebmeier and McRoy, 1989), respectively, and amphipods and bivalves were the dominant taxa (Grebmeier and McRoy, 1989; Grebmeier and Cooper, 1995; McCormick-Ray et al., 2011). In particular, Ampeliscid amphipods dominated in vast areas of the northern Bering Shelf, with high values of mean abundance and biomass at 4,606 ind./m² and 263.5 g/m² (the conversion factor of wet weight to dry weight is 0.2) (Highsmith and Coyle, 1990), respectively, and were the primary prey of the migratory California gray whale. However, compared with results obtained from previous studies, the macrobenthic communities of the Bering Sea have undergone significant structural changes.

Despite amphipods remained one of the dominant groups, a significantly reduced abundance was exhibited in the northern Bering Shelf. A shift in gray whale feeding sites was reported from the northern Bering Sea to Barrow off of the northern slope of Alaska (Moore et al., 2003, 2006). Furthermore, the abundance of mollusks was significantly decreased, and no longer a major group in the northern Bering Shelf. The results showed that the average abundance of macrobenthos in the northern Bering Shelf significantly declined (1,028 ind./m²) in recent decades, whereas the biomass significantly increased (1,435.4 g/m²). In addition, this similar trend was found in the Bering Slope compared with the results of an investigation conducted in the summer of 1999 (Lin et al., 2016). Recent studies indicate a decline in the organic carbon supply and bivalve biomass, which has been interpreted as a consequence of seasonal sea ice retreat and increasing temperatures (Grebmeier and Dunton, 2000; Lovvorn et al., 2003; Grebmeier et al., 2006b). All of these changes support the hypothesis that a large-scale ecosystem change is underway.

5 Conclusions

Based on multiyear biological, chemical, hydrological and geological surveys, this study analyzed the community structure and the temporal and spatial patterns of the macrobenthos in the Bering Sea and their relationships to environmental factors. The macrobenthic communities of this region were dominated by northern cold-water species and immigrant eurythermic species, and exhibited a dispersed and patchy distribution pattern. Combined influenced by the sediment type, water depth, and currents, the northern Bering Shelf and Bering Slope exhibited a high-standing biomass. In recent decades, the macrobenthos of the Bering Sea has undergone significant structural changes, exhibiting a decline in abundance and an increase in biomass. In addition, amphipods and bivalves have been gradually replaced by other taxa, indicating that these benthic ecosystems have been undergoing large-scale structural changes, likely due to seasonal ice melting; changes in organic carbon input; and climate change.

Acknowledgements

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An ecological survey of the abundance and diversity of benthic macrofauna in Indonesian multispecific seagrass beds

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Abstract
Seagrasses are one of the most productive ecosystems in coastal areas and support a wide variety of associated fauna. The tropical Indo-Pacific region is considered to have the highest diversity of seagrass plant species and the largest distribution areas of seagrass, yet the seagrass macrofauna in this region are poorly understood. To help fill this gap in our knowledge, an ecological survey was conducted to describe the abundance and diversity of benthic macrofauna from tropical seagrass beds and to determine between-station variations within a transect and between-site variations in macrofaunal abundance, taxa richness and community structure. Benthic macrofaunal samples associated with seagrass beds were collected with a core sampler on the east coast of North Sulawesi in May 2014 and on the west coast in October 2015. A total of 149 species from 14 higher taxa was collected. The most species-rich groups were polychaetes (56 species, 26% of total individual numbers), decapods (20 species, 9% of total numbers) and amphipods (18 species, 35% of total numbers). Between-station variations within a transect displayed different patterns between the east coast and the west coast. On the east coast, there were marked variations in abundance between stations within a transect for the macrofauna and amphipod assemblages. Both taxa richness and abundance varied with station for the macrofauna and polychaete assemblages on the west coast, resulting from the heterogeneity of the substrate along a transect. One-way ANOSIM together with MDS ordination indicated that macrofaunal community structure in seagrasses differed significantly between the east coast and the west coast, corresponding with the division of seagrasses into two broad categories of habitats, i.e., mangrove-seagrass-reef continuum and seagrass-reef continuum. Compared with other studies in tropical areas, the abundance and diversity of benthic macrofauna in the present study were moderate. The reason for the two markedly distinct macrofaunal communities might be attributed to multiple factors, including sediment pattern, seagrass structure and temporal changes.

Key words: benthic macrofauna, diversity, abundance, community structure, seagrass bed, North Sulawesi


1 Introduction
Seagrasses are marine flowering plants that are commonly found in shallow coastal waters around the world (Green and Short, 2003). Unlike mangroves and coral reefs with distribution restricted to tropical regions, seagrasses can extend their distribution from tropical regions to temperate regions, except Antarctica (den Hartog, 1970). Seagrasses exhibit low taxonomic diversity with only approximately 60 species worldwide (Saenger et al., 2013), and the highest diversity of seagrass plant species is centered in the tropical Indo-Pacific (Waycott et al., 2004). Despite their limited diversity, seagrasses are one of the most productive ecosystems (Duarte and Chiscano, 1999; Short et al., 2007) and have enormous ecological and economic values, including organic carbon export to adjacent ecosystems, food provision, habitat for associated fauna, sediment stabilization, shoreline protection, etc. (Fonseca, 1989; Eyre and Ferguson, 2002; Orth et al., 2006; Heck et al., 2008). Despite their importance, the scientific community now realizes that seagrasses are declining globally at an unprecedented rate, especially in the Indo-Pacific, due to increases in natural and anthropogenic disturbances (Orth et al., 2006; Waycott et al., 2009).

Seagrasses enhance biodiversity (Leopardas et al., 2014). A wide variety of benthic macrofauna are attracted to seagrass vegetated areas, including epifauna on the leaves of the seagrass and infauna in the surface sediments (Williams and Heck, 2001; Tanner, 2005; Klumpp and Kwak, 2005). Among all macrofaunal assemblages, polychaete worms, molluscs and crustaceans (mainly amphipods and decapods) are the dominant fauna, and echinoderms, sipunculids, and nemertean worm species are common (Adulyanukosol and Poovachiranon, 2006). Benthic macrofauna play an important ecological role in a detritus-based food web of seagrass, forming a trophic linkage between primary producers and higher trophic-level predators (Klumpp et al., 1989; Orth et al., 1984). They also affect the physical structure of seagrass habitats and growth of seagrass species through burrowing activity (Orth et al., 1984; Valentine et al., 1994). Analysis of benthic mac-

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rofauna is very important to facilitate future monitoring studies of seagrass beds, because the loss and deterioration of seagrasses will eventually lead to the alteration of macrofaunal communities. Quite a few studies have been conducted on the abundance, diversity and seasonality of seagrass macrofauna and the impact of seagrass structure (Orth et al., 1984; Mukai et al., 1999; Somerfield et al., 2002; Klumpp and Kwak, 2005), but most of the studies were focused on temperate seagrass beds instead of tropical (Klumpp and Kwak, 2005).

The coastal areas in North Sulawesi, Indonesia, are characterized by large seagrass beds, often constituted by multiple species. Little is known about seagrasses throughout the area, even the basic information, such as species composition of seagrass plants and the extent of seagrass beds. The seagrass beds here can be divided into the following two broad categories of habitats: seagrass+reef continuum on the east coast and mangrove+seagrass+reef continuum on the west coast. Seagrass beds in the former habitat are located between the coastline and submerged reefs, while those in the latter are situated between mangroves and fringing reefs. The present study represents the first attempt to describe seagrass macrofauna in this area, also serves as part of a wider joint study aimed at understanding marine biodiversity and the environment in Indonesian tropical areas. The aims of this study were (1) to describe the diversity of seagrass macrofauna, (2) to determine whether the taxa richness and abundance of seagrass macrofauna vary with station within a transect and (3) to compare benthic macrofaunal communities between the two categories of seagrass beds.

2 Materials and methods

2.1 Study area

The survey area was located in North Sulawesi (Fig. 1) and supports diverse coastal ecosystems, including mangroves, coral reefs and seagrasses. The tidal range in this region was approximately 1.5 m based on personal observation. Fieldwork on seagrasses was carried out at Site 2 (on the east coast of North Sulawesi) in May 2014 and at Site 1 (on the west coast) in October 2015. We sampled two transects (SGT1 and SGT3) on the east coast, with an inshore station and an offshore station along one transect (an extra station was located between the inshore station and offshore station at the SGT3 transect). Fine sand dominated the seagrass bed. There were eight seagrass species, dominated by *Thalassia hemprichii*, *Halodule pinifolia* and *Cymodocea rotundata*. In the seagrass bed along the west coast, we established three transects with two sampling stations within each transect. Inshore stations were located near the edge of the mangroves while offshore stations were approximately 20 m from the edge of the reef. The substrate here is heterogeneous. Inshore stations are influenced by mangrove detritus while small coral rubble is scattered around seagrass beds at offshore stations. Seagrass beds along the west coast consisted of six species, dominated by *Syringodium isoetifolium* and *Thalassia hemprichii*.

2.2 Collection and measurement of benthic macrofauna

All field sampling was conducted at low tide. Four or five replicate core samples of sediment were collected randomly within a chosen 10 m × 10 m quadrant at each station, using a PVC core sampler with an inner diameter of 10 cm (78.54 cm² surface area for each core sample). The core sampler was pushed into the sediment to a depth of 30 cm. Next, the sediment samples were sieved through 0.5 mm mesh screen in the field, and specimens retained on the sieve were preserved in 7% diluted seawater formalin for further analysis. In the laboratory, living organisms were extracted under a microscope, identified to the lowest possible taxon and counted. In the present study, macrofaunal abundance was reported as the number of individuals per square meter, and taxa richness was expressed as the number of species occurring in one core sample.

2.3 Statistical analyses

To characterize the functional composition of the fauna, each macrofaunal species was assigned to a microhabitat category and feeding guild using information from the literatures. Habitat categories (Rainer and Fitzhardinge, 1981; Klumpp and Kwak, 2005) were as follows: (1) infaunal tubicolous, (2) infaunal burrower, (3) infaunal commensal, (4) epifaunal domicolous and (5) epifaunal free-living. All fauna were divided into five feeding guilds (Boaventura et al., 1999), i.e., (1) carnivorous group, (2) omnivorous group, (3) detritivorous group, (4) phytophagous group and (5) planktophagous group.

To test between-station variations within a transect in the abundance and taxa richness of the macrofauna and major
groups, nested ANOVA was applied. The analysis was also carried out to detect the difference in abundance and taxa richness of macrofauna between the two sampling sites. Prior to the ANOVA, data were log_{10}(X+1)-transformed if they did not meet the assumptions of normality and homogeneity required for the ANOVA test.

The community structure of seagrass macrofauna was analyzed by means of PRIMER 6.0 software, using cluster analysis and non-metric multidimensional scaling (MDS). The cluster analysis was based on the Bray-Curtis similarity index (Bray and Curtis, 1957) and used the group average method. The similarity matrix was based on square-root transformed abundance. The ANOSIM routine (Clarke and Warwick, 1994) based on average abundances was used to analyze the between-site variation. Macrofaunal species responsible for the observed patterns were identified by means of the SIMPER routine (Clarke and Warwick, 1994).

3 Results

3.1 Macrofaunal diversity

In total, 824 specimens of benthic macrofauna representing 149 species were collected from the seagrass beds of North Sulawesi (Table 1). Of these, 14 higher taxa were identified as follows: Polychaeta, Bivalvia, Gastropoda, Tanaidacea, Amphipoda, Decapoda, Ophiuroidea, Echinoida, Holothurioidea, Porifera, Cnidaria, Nemertinea, Sipuncula and Chordata. The major macrofaunal groups ranked by species numbers were polychaetes (38%), decapods (33%) and amphipods (28%); however, ranking by the number of individuals, the order was amphipods (35%), polychaetes (26%) and tanaids (17%).

Polychaetes showed the highest species diversity in the survey area, represented by 56 species, of which the most abundant species in decreasing order were Ceratoneireis mirabilis, Linophorus cirratus and Dasybranchus caducus. Decapods (20 species) were the next richest group, and the most abundant species were Callianassa sp. and Eualus sp. Amphipods were the most abundant group with 18 species, among which the most abundant were Ampelisca miharaensis, Melita longidactyla, Grandidierea sp.1 and Photis hawaiiensis. Although only two species of tanaids were recorded, the paratanaid species Chondrochelia dubia was found in great number.

In the case of microhabitat type, 43 species (29%) of benthic macrofauna were categorized as epifaunal free-living, 50 species (34%) as epifaunal domicolous and 42 species (28%) as infaunal burrower. Polychaetes consisted of 54% epifaunal domicolous and 29% infaunal burrower. Eighty-five percent of decapods were categorized as epifaunal free-living, and most amphipods were composed of 50% epifaunal domicolous and 28% infaunal burrower. For feeding guild, 55 species (36.9%) had a diet of invertebrates, 42 species (28.2%) were detritivorous, 19 species (12.8%) were planktivorous, 19 species (12.8%) were omnivorous, and 14 species were found to be phytophagous. Hence, most of the benthic macrofaunal species feed on invertebrates and organic debris on the sediment. For the major groups occurring in the seagrass, most polychaetes were composed of carnivores and detritivores, amphipod species mainly fed on plankton, and decapod species were mainly classified as either carnivore or omnivore.

3.2 Abundance and taxa richness of macrofauna

In the seagrass bed along the west coast of North Sulawesi, the taxa richness of benthic macrofauna varied with sampling station (nested ANOVA, $F=10.805$, $P<0.001$), which tended to be higher at offshore stations than at inshore stations along each transect (Fig. 2, Table 2). There was also significant variation between transects (nested ANOVA, $F=5.068$, $P=0.018$). Further analysis of the major groups revealed that the taxa richness of the polychaete assemblage differed significantly between stations within a transect (nested ANOVA, $F=7.269$, $P=0.002$). Variation in macrofaunal abundance was statistically significant between stations within a transect (nested ANOVA, $F=3.401$, $P=0.04$), with abundance usually higher at offshore stations except at the WR1 transect. However, macrofaunal abundance did not differ between transects. Further analysis of the major groups showed that there were marked variations in polychaete abundance between stations within a transect (nested ANOVA, $F=5.646$, $P=0.007$) and in amphipod abundance between transects (nested ANOVA, $F=3.788$, $P=0.043$).

Compared with the seagrass bed on the west coast, benthic macrofauna displayed a different distribution pattern in the seagrass bed along the east coast. There was no marked difference in the taxa richness of macrofauna between transects and between stations within a transect. However, the taxa richness of the amphipod assemblage differed significantly between transects (nested ANOVA, $F=12.398$, $P=0.003$). The nested ANOVA revealed that macrofaunal abundance varied with transect (nested ANOVA, $F=8.345$, $P=0.01$) and station (nested ANOVA, $F=3.776$, $P=0.03$). Higher macrofaunal abundance occurred at the inshore station than at the offshore station at the SGT1 transect, although this trend was opposite at the SGT3 transect, where abundance was higher at the offshore station. Further analysis of the major groups indicated that there were significant differences in amphipod abundance between transects and between stations within a transect, but not in polychaete abundance (Table 2).

Comparison of the taxa richness and abundance of benthic macrofauna between the two study sites is shown in Fig. 3. The taxa richness of benthic macrofauna in seagrass beds was from (8.1±3.9) to (9.5±4.8) species/core on the east coast while (9±3.0) to (12.3±7.6) species/core on the west coast. Macrofaunal abundance ranged between (1 602±784) ind./m$^2$ and (2 725±1 357) ind./m$^2$ on the east coast and between (2 228±1 363) ind./m$^2$ and (2 722±1 603) ind./m$^2$ on the west coast. The nested ANOVA revealed that neither taxa richness (nested ANOVA, $F=0.961$) nor macrofaunal abundance (nested ANOVA, $F=0.362$, $P=0.551$) differed significantly between the two study sites.

3.3 Macrofaunal community structure

Superimposed Bray-Curtis clusters on the MDS ordination (Fig. 4) showed that all the sampling stations can be divided into two groups at a similarity level of 20%, in agreement with the division of the two broad categories of seagrass habitats, i.e., one habitat on the east coast and the other on the west coast. ANOSIM confirmed the differences in community structure between the two habitats to be significant (global $R=0.944$, $P=0.002$). The SIMPER analysis showed the highest dissimilarity (>2% dissimilarity contribution) to be as result of Ampelisca miharaensis, Chondrochelia dubia, Melita longidactyla, Ampelisca bocki, Photis hawaiiensis, Paraphoxus tomokaensis and Grandidierea sp.1. These species were from the amphipod and tanaid assemblages. Furthermore, the classification of the sampling stations varied between the two study sites at a similarity level of 30%, as shown in Fig. 4. Macrofaunal communities were more similar between stations within the same transect on the east coast. However, it was not the case on the west coast, where sampling stations at the same position (inshore stations or
<table>
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<th>Species</th>
<th>n</th>
<th>Percentage/%</th>
<th>H.T.</th>
<th>F.G.</th>
<th>Species</th>
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*Table 1. Benthic macrofauna occurring in the seagrass beds of North Sulawesi*
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Note: Individual numbers of macrofauna are summed across all the samples. The character “–” in the second column represents no statistics of individual number for corresponding species, as they are impossible to be counted. n is individual number. H.T. (microhabitat type): It represents infaunal tubicolous, Ib infaunal burrower, Ic infaunal commensal, Ed epifaunal domicolous, and Ef epifaunal free-living.
F.G. (feeding guild): C represents carnivorous, O omnivorous, D detritivorous, Ph phytophagous, and Pl planktophagous.

4 Discussion and conclusions

4.1 Macrofaunal diversity and abundance

Although seagrasses are widely distributed in the shallow coastal waters of North Sulawesi, our knowledge of the seagrasses and associated fauna in this area remains poor, because no research papers were available in the database. Considering that the survey area is known for its high biodiversity, the finding that 149 species from 14 higher taxa were collected from seagrass beds of North Sulawesi indicated that the diversity of benthic macrofauna in the present study is moderate. The overall species diversity recorded in this study was greater than that reported for other areas in the tropical Indo-Pacific. For example, Klumpp and Kwak (2005) recorded 110 species in the Cockle Bay, North Queensland, Australia. Nakaoka (2001) found 61 species in an intertidal flat in Thailand. Forty-five species were recorded at offshore stations) clustered together.

Lopez Jaena, southern Philippines (Leopardas et al., 2014). However, the other studies cited above all referred to one site, which is in contrast to the two sites included in our study. The most speciose group of the present study was polychaetes, followed by decapods and amphipods. Some or all of these groups also dominate seagrass beds in other areas of the Indo-West Pacific. For example, polychaetes, amphipods and mollusks are the dominant groups in the Princess Royal Harbour, Albany, Western Australia (Hutchings et al., 1991); polychaetes and amphipods in the Western Port, Thomas Bay, Western Australia (Mukai et al., 1999); polychaetes and mollusks in the Haad Chao Mai National Park, Thailand (Nakaoka, 2001); polychaetes and amphipods in the Cockle Bay, Australia (Klumpp and Kwak, 2005); and polychaetes and mollusks, at Lopez Jaena, southern Philippines (Leopardas et al., 2014). In the present study, mollusks are the second most speciose group when bivalves (10 species) and gastropods (17 species) are considered together.

The mean abundance of seagrass macrofauna was 2 051
ind./m$^2$ on the east coast and 2,435 ind./m$^2$ on the west coast. Compared with other tropical seagrass beds, the benthic macrofauna of the present study has considerably higher abundance, e.g., 654 ind./m$^2$ was recorded in the Apalachee Bay, Florida (Lewis III and Stoner, 1983), 31.8–64.7 ind./m$^2$ was observed at Lopez Jaena, southern Philippines (Leopardas et al., 2014) and 751–1,133 ind./m$^2$ was reported for southwest Sulawesi, Indonesia (Vonk et al., 2010), although values lower than 5,800–8,300 ind./m$^2$ observed in Kuraburi, Thailand (Whanpetch et al., 2007) and 28,148 ind./m$^2$ in the Cockly Bay of Australia (Klumpp and Kwak, 2005).

Table 2. $F$ values and level of significance for the nested ANOVA of the taxa richness and abundance of benthic macrofauna in the seagrass beds of North Sulawesi.

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<td>Between stations</td>
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<td>$P$</td>
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Note: Level of significance: $^1$0.05; $^2$0.01; $^3$0.001.

Fig. 2. Variation in the taxa richness and abundance of benthic macrofauna between transects and between stations within a transect in the seagrass beds of North Sulawesi.

4.2 Variations between stations within a transect

Previous studies have reported the effect of seagrass structure on associated fauna, including seagrass biomass (Orth et al., 1984; Klumpp and Kwak, 2005), small-scale spatial heterogeneity of the seagrass vegetation (Nakaoka, 2001), seagrass meadows with different canopy structures (Vonk et al., 2010) and vegetation dominated by different seagrass species (Leopardas et al., 2014). However, sediment patterns in seagrass beds have been seldom mentioned in the literature. It is well known that sediment type plays an important role in macrofaunal community (Rhoads, 1975). In the present study, there was an important
**Fig. 4.** MDS ordination of seagrass macrofauna at 11 sampling stations in North Sulawesi, Indonesia. Ordination is superimposed with Bray-Curtis similarity clusters at the 20% (solid lines) and 30% (dotted lines) level. The sampling station code is expressed as transect + position of the station along a transect.

Finding that spatial variations in seagrass macrofauna displayed distinctly different patterns across the two study sites, which might be related to the different sediment patterns in the seagrass beds. On the east coast, the substrate was consistent throughout the study area. The vast beach extends from the coastline to the subtidal area along the investigated transect, and seagrasses grow in a sediment composed of fine sand. The homogenous substrate and similar species composition of seagrasses resulted in comparable communities between the stations within a transect. Additionally, the nested ANOVA showed that no variations in abundance and taxa richness between stations within a transect were found in the surface sediment-inhabiting polychaete assemblage, which could also be explained by the homogenous sediment pattern. We also observed that amphipod abundance varied with transect and with station within a transect. The great number of amphipods at the inshore station of the SGT1 transect might explain the spatial variation that was recorded on the east coast. Amphipods were small opportunistic species and they were usually recorded in large numbers at random stations where abundant food was available.

However, on the west coast, seagrasses were influenced by nearby habitats such as mangroves and coral reefs. A heterogenous sediment pattern was formed along a transect with coral rubber scattered at offshore stations. Therefore, the communities of benthic macrofauna were less similar between stations within a transect than between stations at the same position, as revealed by the MDS ordination. At inshore stations near mangroves, mangrove detritus covered the nearby seagrass beds, which might reduce the water transparency. Consequently, the epiphytes on the leaves of seagrass and the seagrass itself were not able to fully photosynthesize, and food supply was limited for associated fauna. In contrast, at offshore stations in reef flats, the presence of coral rubble in the sediment increases the complexity of the benthic environment and provides more microhabitats and shelters for benthic fauna, especially for polychaete assemblages. This might explain the higher abundance and taxa richness of polychaetes at offshore stations.

### 4.3 Variations between the two study sites

In the present study, all the sampling stations can be divided into two groups at a similarity level of 20%. The division was in agreement with the following two broad categories of habitats: reef-seagrass continuum on the east coast and reef+seagrass+mangrove continuum on the west coast. Although there were no marked variations between the two study sites in terms of the abundance and taxa richness of benthic macrofauna, the difference in community structure was confirmed by the ANOSIM to be significant ($P=0.002$). The difference in macrofaunal community structure between the two study sites might be attributed to multiple reasons. First, there is a marked difference in sediment pattern. The complex substrate on the west coast is a combination of mangrove detritus, coral rubble and sand, which is different from the fine sand on the east coast. Previous studies have shown that sediment characteristics have a great impact on the benthic fauna living in the sediment (Rhoads, 1975; Snellgrove and Butman, 1994). Second, the species composition of seagrass varied between the two sites. The seagrass vegetation was dominated by *Thalassia hemprichii*, *Halodule pinifolia* and *Cymodocea rotundata* on the east coast, while *Syringodium isoetifolium* and *Thalassia hemprichii* dominated the west coast. Klumpp and Kwak (2005) found that the relative proportion of dominant amphipods differed among the three most abundant seagrass species, and tanaids and isopods with stout bodies tended to be abundant on the wide-bladed seagrass. However, Leopards et al. (2014) observed that the species composition of macrofauna did not vary significantly by vegetation type. Third, temporal changes in seagrass macrofauna may exist. Our fieldwork on the east coast was conducted in October 2014, while on the west coast, we worked in May 2015. Temporal changes in macrofauna have been recorded in other studies. Bos et al. (2008) observed a lower abundance of the sea star *Protoreaster nodosus* during certain months of the year. Klumpp and Kwak (2005) found that the temporal pattern of macrofaunal abundance correlated with temporal variations in seagrass biomass in the Cockle Bay. The present study showed that there was significant difference between the two categories of seagrass habitats in the benthic macrofaunal community and biological environment. More studies are needed to analyze how nearby habitats such as mangroves and coral reef affect biodiversity in seagrass.

### References


Eyre B D, Ferguson A J P. 2002. Comparison of carbon production and decomposition, benthic nutrient fluxes and denitrification in seagrass, phytoplankton, benthic microalgal- and macroal-


Inferring trophic variation for Antarctic krill (Euphausia superba) in the Antarctic Peninsula from the austral fall to early winter using stable isotope analysis

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Abstract

The Antarctic krill (Euphausia superba) is a key species in the Southern Ocean ecosystem and an important link in the food web of the Antarctic ecosystem. The trophic information for this species during the transition from the austral fall to the winter is important to understand its poorly known overwintering mechanisms. However, the few studies on the topic differ in their results, in terms of both spatial and temporal variables. We investigated the size dependence and monthly and regional variation in δ13C and δ15N values of adult krill in the Antarctic Peninsula, in the austral fall (April to May) and the early winter (June). We aimed to examine the trophic variations of krill occurred during this period, and the relationship between krill and their feeding environment in the Antarctic marine ecosystem. The following results were obtained: (1) no significant relationship was observed between size and the δ13C value of krill, but the δ15N value of krill presented a remarkable association with size; (2) the δ13C values of krill increased during the austral fall, but no remarkable variation existed at the onset of winter, and the δ15N values were not significant different during this period; (3) mean δ13C values of krill differed significantly between the Bransfield Strait and the South Shetland Islands. Our data imply that adult krill present size-, season-, and region-dependent trophic variation during the transition from austral fall to early winter in the Antarctic Peninsula.

Key words: Euphausia superba, stable isotope, trophic variation, Antarctic Peninsula, feeding habit


1 Introduction

Antarctic krill (Euphausia superba Dana, 1850; hereafter, krill) is the most important link in the Antarctic food web, and is considered a key species of Antarctic ecosystem (Everson, 2000) because of its high biomass and importance as a prey for penguins, flying birds, marine mammals, fishes, and benthic invertebrates (Atkinson et al., 2009). It is important to understand the trophic condition of krill in hotspots such as the South Shetland Islands and the Antarctic Peninsula (Kokubun et al., 2015), given the significant warming in the region (Atkinson et al., 2004; Stowasser et al., 2012; Kokubun et al., 2015), coupled with increasing krill-fishing operations (Nicol and Foster, 2016).

Considering the important role of krill in the Southern Ocean food web, its feeding habits, particularly the diet of krill in the austral summer, have been widely studied (Barkley, 1940; Kils, 1983; Hopkins, 1985; Croxall et al., 1999; Schmidt and Atkinson, 2006). More recently, biomarker analyses have been introduced to complement the conventional dietary analysis and to understand the diet and trophic levels of animals, and stable isotope analysis is one of the promising approaches that has emerged as a valuable tool for examining trophic relationships (e.g., Michener and Schell, 1994). Stable nitrogen isotope (δ15N) values indicate the trophic position of a consumer (Vander Zanden and Rasmussen, 2001), whereas stable carbon isotope (δ13C) values can be used as a proxy for the source of primary production, as well as the inshore and benthic versus offshore and pelagic feeding preferences of a consumer (Hobson and Welch, 1992; France, 1995). δ13C and δ15N analysis has developed into a major approach for determining the source of food in pelagic/benthic systems and the trophic structure of marine organisms in marine food webs (Wada et al., 1987). Food source (Frazer, 1996; Schmidt et al., 2006), ontogenetic niche expansion (Polito et al., 2013), and trophic relationships of krill (Schmidt et al., 2003; Corbisier et al., 2004; Stowasser et al., 2012; Kokubun et al., 2015) have been examined in different regions of the Southern Ocean using stable isotope analysis. The extant studies indicate spatial-temporal and ontogenetic differences in the food source and the trophic relationships of krill. However, the trophic status of krill
during the austral late fall to early winter has received little attention and the trophic variation of krill during the onset of austral winter is very important to understand the overwintering mechanism of this species. Therefore, the objectives of the present study were: (1) to understand the trophic status of krill in the Antarctic Peninsula, and (2) examine the monthly (seasonal) differences (if any) in the food sources of krill, particularly during the austral late fall to early winter. The results would be important in developing a comprehensive understanding of the relationship between krill and their feeding environment in the Antarctic marine ecosystem, particularly during the transition from austral fall to early winter, when krill prepare for the long-term overwin-
tering period.

2 Materials and methods

2.1 Survey area and sampling

Krill samples were collected randomly by Chinese scientific observers aboard a large-scale trawler, Kaili (total of length, 120.7 m; gross registration tonnage, 7 847 t; main engine 5 296 kW; condenser mesh size 20 mm), from May 26 to June 7, 2012, and April 9 to May 28, 2015. The sampling areas were located off the South Shetland Islands (between Snow Island and Rugged Island, SSI) and in the Bransfield Strait (BS) (Fig. 1).

2.2 Sample preparation and stable isotopic analyses

In the laboratory at the Shanghai Ocean University (SHOU), individual krill were homogenized and freeze-dried for 48 h in an oven at −60°C, after measuring their total length (from the tip of the rostrum to the tip of the uropod, TL, mm) and wet weight (WW, g), examining the sex, and removing the head/shell. Sex and the maturity stage were identified following Makarov and Denys (1981). The samples were analyzed using a system that coupled an elemental analyzer (Elementar Vario Cube CN series) with a continuous-flow isotope ratio mass spectrometer (CF-IRMS; Isoprime, GV Instruments, U.K.). All isotope analyses were carried out at the Laboratory of Stable Isotope Analysis, SHOU. Stable isotope abundance was expressed in delta (δ) notation, as the magnitude of deviation from the conventional standard Peedee Belemnite (PDB) for carbon and air N₂ for nitrogen, in parts per thousand (%), according to the equation: δX=(Rsample/

\[\text{R}_{\text{standard}}-1] \times 10^3, \text{ where } X = ^{13}\text{C} \text{ or } ^{15}\text{N}, \text{ and } R \text{ is the } ^{13}\text{C}/^ {12}\text{C} \text{ or } ^{15}\text{N}/^ {14}\text{N} \text{ ratio, respectively (Fry and Sherr, 1989). Sucrose (ANU } \text{C}_{12}\text{H}_{22}\text{O}_{11}; \text{ Gaithersburg, MD) and ammonium sulfate } ([\text{NH}_4]_2\text{SO}_4; \text{ NIST) were used for the internal } ^ {13}\text{C} \text{ and } ^ {15}\text{N} \text{ calibration, respectively. Sample precision was } 0.1\% \text{ and } 0.2\% \text{ for the } ^ {13}\text{C} \text{ and } ^ {15}\text{N} \text{ values, respectively.}

2.3 Statistical analysis

Analysis of variance (ANOVA) was performed to determine the monthly difference in size distribution. Analysis of covariance (ANCOVA) was used to determine the effect of sex on weight-length relationship of krill. To investigate the relationships of size with δ¹³C and δ¹⁵N values of krill, linear regressions analyses were conducted. Size-, monthly- and regional-differences in δ¹³C or δ¹⁵N values of krill were examined using Student’s t-test for two groups or ANOVA for three or more groups. All the tests were two-tailed with a significance level of p<0.05. Data are presented as mean±SD, unless stated otherwise. Statistical analyses were carried out using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

3 Results

3.1 The relationship between weight and length

The TL ranged from 35.10 mm to 58.93 mm ((48.19±9.06) mm) and the wet weight ranged from 0.180 2 g to 1.438 3 g ((0.774 7±0.365 8) g). The length distribution showed no monthly difference (ANOVA; F=2.64, p=0.08, n=52). Sex differences could not be found for the weight-length relationship within the samples (Fig. 2) (ANCOVA; F=3.909, p=0.05), which can be expressed as W=6.753 4×10⁻⁷TL¹³⁵₆₄⁺ (R²=0.972 1, p<0.01, n=52) for the combined samples.

3.2 The relationship of size with δ¹³C and δ¹⁵N of krill

The δ¹³C and δ¹⁵N values of krill ranged from −30.0‰ to −0.0‰ (−26.3‰±1.65‰) and 3.0‰ to 5.6‰ (4.6‰±0.62‰). The differences between females and males were not detected for δ¹³C (Student’s t-test; t=0.494 7, p=0.623 3) and δ¹⁵N (Student’s t-test; t=0.471 5, p=0.639 6) values of krill, so the samples were combined. There was no significant relationship between size and the δ¹³C value (R²=0.000 7, p=0.856 8, n=52) of krill (Fig. 3). However, the δ¹⁵N value of krill demonstrated a significant positive correlation with size (R²=0.172 4, p=0.002 2, n=52) (Fig. 4).

3.3 The monthly variation in δ¹³C and δ¹⁵N of krill

The mean δ¹³C values of krill were −27.2 (±1.06)‰, −25.9 (±1.54)‰, and −26.3 (±2.21)‰ in April, May and June, respectively. A weak monthly difference was found for the mean
$\delta^{13}C$ values across the three months (ANOVA; $F=3.19$, $p=0.05$) (Fig. 5). A significant increase in the $\delta^{13}C$ values was observed from April to May (Student’s $t$-test; $t=2.002$, $p=0.007$), but no significant variation existed from May to June (Student’s $t$-test; $t=2.03$, $p=0.007$). They were not significantly different from April to May either (Student’s $t$-test; $t=2.02$, $p=0.015$) or from May to June (Student’s $t$-test; $t=2.03$, $p=0.043$).

### 3.4 The regional variation in $\delta^{13}C$ and $\delta^{15}N$ of krill

The mean $\delta^{13}C$ values of krill were -26.6(±1.59)%o, 25.8(±1.66)%o in the BS and off the SSI, respectively (Fig. 6). The mean $\delta^{15}N$ values of krill were 4.2(±0.56)%o, 4.6(±0.66)%o, and 4.8(±0.51)%o in April, May, and June, respectively. The $\delta^{15}N$ values showed a slight increase from April to June (Fig. 5), although there were not statistically significant (ANOVA; $F=1.97$, $p=0.15$). They were not significantly different from April to May either (Student’s $t$-test; $t=2.02$, $p=0.15$) or from May to June (Student’s $t$-test; $t=2.03$, $p=0.043$).

### 4 Discussion

#### 4.1 The relationship of trophic position with krill size

The present study indicated that the $\delta^{13}C$ value of krill had no relationship with size, but a significant relationship was inferred between the $\delta^{15}N$ value and size. Similar results were obtained by Frazer (1996), who observed no statistical relationship between $\delta^{13}C$ value and size, but a weak positive correlation between $\delta^{15}N$ value and size of larval krill in the austral winter, and Polito et al. (2013), who reported that, with ontogenetic niche expansion, adult krill have higher and more variable $\delta^{15}N$ values but consistent $\delta^{13}C$ values than juveniles do during the austral summer around the SSI. This indicated that both adults and juveniles were feeding on phytoplankton, but the adults also fed on prey from higher trophic levels (Polito et al., 2013). Stowasser et al. (2012) also reported a positive and significant correlation between $\delta^{15}N$ values and body mass of krill. This was also supported by Agersted et al. (2014), who found that the largest species, Meganyctiphanes norvegica, had the highest trophic position in the Arctic Ocean. The swimming capability of krill at different stages can partly explain the ontogenetic differences in size and food requirements. Larger krill, with their stronger swimming capabilities (Huntley and Zhou, 2004), can explore a wider range of habitats, which increases the ability to encounter motile prey (Schmidt and Atkinson, 2016).

#### 4.2 The monthly (seasonal) trophic variation of krill

The $\delta^{13}C$ and $\delta^{15}N$ of krill did not show a monthly variation in the present study, suggesting that the diet had no significant effect during the transition from austral fall to early winter; however, larger individuals had higher $\delta^{15}N$ values. A possible explanation is that phytoplankton likely remained an important food resource from austral summer to early winter, but food items became more scarce and krill resorted to more opportunistic or carnivorous feeding after the end of phytoplankton blooms during the austral early winter, and larger individuals had a strong capacity to find food sources and feed on prey at
higher trophic positions (Schmidt and Atkinson, 2016). A significant increase in the δ³¹C values was observed from April to May, but no remarkable variation existed from May to June, when the effect of monthly length can be neglected, suggesting that primary production increased in the austral fall, but remained stable during the transition to early winter. Meyer et al. (2010) also indicated that, compared with the austral winter, the carbon content of krill was higher in the austral fall; however, the nitrogen content was relatively stable from the austral fall to winter. A plausible explanation is that, in the transition period before the onset of austral winter, krill was still physiologically active but feeding was slow due to a winter close down (Atkinson et al., 2002). From Fig. 7, it can be seen that the trophic position of krill varied regionally, if the effect of the sampling process is not considered. For the SSI, the trophic variation of adult krill in the austral late fall to early winter was significant than that in the austral summer (Fig. 7 and Table 1), implying that the food sources of krill were similar in the summer; however, krill would feed on food items with wider trophic positions in the austral fall. Furthermore, the samples in the present study were collected during two years; although a sampling month overlapped (May), the potential effect of inter-annual differences on seasonal variation needs to be considered.

4.3 The regional trophic variation of krill

No significant difference was observed on mean δ³¹C values between the BS and the SSI, but krill in the SSI presented significantly higher δ¹⁵N values than krill in the BS during the austral fall to early winter, indicating that the food sources of krill were not different, but food items with higher trophic positions were available in the SSI. From Fig. 1, it can be seen that the krill sampled in the SSI were closer to the shore than those in the BS. Given the similar foraging habitats between the two regions, krill closer to the shore consumed food sources at higher trophic positions during the austral fall to early winter (Nishino and Kawamura, 1996). Based on the stomach content analysis of krill-feeding penguins, Kokubun et al. (2015) indicated that δ¹³C values of krill did not differ between on-shelf and off-shelf trips, but krill from off-shelf trips had higher δ¹⁵N values than those from on-shelf trips during the austral summer (December to January). Although Kok-

Table 1. The δ³¹C, δ¹⁵N values of krill in the different regions of the Southern Ocean

<table>
<thead>
<tr>
<th>Region</th>
<th>Sampling date</th>
<th>Total length/mm (mean±SD)</th>
<th>Sex/life stage</th>
<th>δ³¹C‰ (mean±SD)</th>
<th>δ¹⁵N‰ (mean±SD)</th>
<th>Source</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>South Shetland Islands</td>
<td>Mar. 2000</td>
<td>unknown</td>
<td>larvae</td>
<td>−25.1±0.9</td>
<td>4.2±1.4</td>
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<tr>
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<td>28.9±3.4</td>
<td>larvae</td>
<td>−26.8±0.7</td>
<td>2.7±0.2</td>
<td>Polito et al. (2013)</td>
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<tr>
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<td>2.4±0.3</td>
<td>Polito et al. (2013)</td>
<td>3</td>
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<td>−28.3±0.7</td>
<td>2.9±0.4</td>
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<td>−26.2±1.0</td>
<td>3.6±0.7</td>
<td>Polito et al. (2013)</td>
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<td>−26.6±1.0</td>
<td>3.3±0.6</td>
<td>Polito et al. (2013)</td>
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<td>adult male</td>
<td>−26.4±1.0</td>
<td>3.5±0.6</td>
<td>Polito et al. (2013)</td>
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<td>Jan. 2009</td>
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<td>3.2±0.4</td>
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<td>4.0±0.24</td>
<td>Kokubun et al. (2015)</td>
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<td>−31.1±0.7</td>
<td>2.1±0.9</td>
<td>Schmidt et al. (2004)</td>
<td>26</td>
</tr>
<tr>
<td>East Antarctic</td>
<td>Jan. 1984</td>
<td>unknown</td>
<td>larvae</td>
<td>−28.1±1.5</td>
<td>1.7</td>
<td>Wada et al. (1987)</td>
<td>27</td>
</tr>
<tr>
<td>Lazarev Sea</td>
<td>Apr. 1999</td>
<td>unknown</td>
<td>adult</td>
<td>−31.3±0.7</td>
<td>3.6±0.4</td>
<td>Schmidt et al. (2004)</td>
<td>28</td>
</tr>
<tr>
<td>East Antarctic</td>
<td>Jan. 1984</td>
<td>unknown</td>
<td>adult</td>
<td>−29.3±0.5</td>
<td>2.7</td>
<td>Wada et al. (1987)</td>
<td>29</td>
</tr>
<tr>
<td>Amundsen Sea</td>
<td>Jan. 2011</td>
<td>46.2±8.10</td>
<td>adult</td>
<td>−25.7±0.5 to 26.2±0.5</td>
<td>5.1±0.3 to 8.9±0.5</td>
<td>Ko et al. (2015)</td>
<td>30</td>
</tr>
<tr>
<td>East Antarctic</td>
<td>Sep. to Oct. 2007/2008</td>
<td>unknown</td>
<td>fucricula</td>
<td>−25.8±0.38</td>
<td>5.6±0.56</td>
<td>Jia et al. (2015)</td>
<td>31</td>
</tr>
<tr>
<td>East Antarctic</td>
<td>Sep. to Oct. 2007/2008</td>
<td>unknown</td>
<td>juvenile</td>
<td>−25.6±0.23</td>
<td>3.1±0.23</td>
<td>Jia et al. (2015)</td>
<td>32</td>
</tr>
<tr>
<td>East Antarctic</td>
<td>Sep. to Oct. 2007/2008</td>
<td>unknown</td>
<td>adult</td>
<td>−26.39±0.66</td>
<td>2.8±0.33</td>
<td>Jia et al. (2015)</td>
<td>33</td>
</tr>
</tbody>
</table>

Note: SD is standardized deviation and code refer to Fig. 7.
The δ15N, δ13C values of krill (Euphausia superba) in the different regions of the Southern Ocean. The numbers derived from Table 1. Blue (red) circle signify the δ13C and δ15N values of krill (Euphausia superba) in the Antarctic Peninsula during the austral summer (fall).

ubun et al. (2015) reported a different result for δ15N values, the samples in their study were collected during the austral summer, where the food sources of krill present a seasonal difference (Meyer et al., 2010). Mordy et al. (1995) demonstrated that the microorganism community under the sea ice, which had high δ15N values, was very important in the food web during the poor phytoplankton abundance in the austral fall–winter. This provides an explanation for the higher δ15N values of krill in the SSI, because sea ice extended gradually to the off-shore region, from the onset of the austral winter, and the microorganisms under the sea ice potentially became the food sources of krill.

The δ13C suggested that the marine food sources originated from benthic or pelagic environments (France, 1995), implying that the krill did not have significantly different feeding selectivity for benthic or pelagic food sources between the austral summer and fall to early winter. However, compared with the BS, there were higher (although statistically insignificant) δ13C values of krill in the SSI. One possible reason is that the samples in the SSI are spatially closer to the sea bottom and the marginal ice-edge zone, and benthic food sources (e.g., benthic diatoms) have higher δ13C values compared to pelagic food sources (e.g., planktonic diatoms) (France, 1995; Kokubun et al., 2015). Another explanation is that krill fed on ice-associated food sources, particularly, ice algae enriched in 13C (Wada et al., 1987; Fischer, 1991).

5 Conclusions

Our study highlights the size- and region-related trophic variation in krill during austral fall to early winter in the Antarctic Peninsula, and provides important data for understanding its overwintering mechanisms, which have been rarely studied. Evidence from the variation in δ13C and δ15N values of krill due to size, month, and region indicate the important role of diet. In order to get more detailed insights into the trophic variation of krill, stable isotopic analysis on different tissues, for example, hepatopancreas, carapace, digestive gland, and stomach, would be a crucial and useful approach for understanding the feeding ecology and overwintering of krill. Although stable isotope analysis is a useful method for evaluating the trophic variation of krill, other methods, such as fatty acid analysis, stomach content analysis, and molecular approach (for example, PCR-DGGE) (Martin et al., 2006), should be also combined to achieve more comprehensive and precise results.

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References


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Size distribution of individuals in the population of Asterias amurensis (Echinodermata: Asteroidea) and its reproductive cycle in China

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Abstract
To obtain baseline information on the size distribution of individuals in the population and reproductive features of sea star Asterias amurensis, monthly surveys of the population were carried out from May to December 2010 and March to May 2015 in coastal waters off Yantai, China. Spawning period was predicted by gonad and pyloric caeca indices as well as anatomical and histological methods. In the A. amurensis population, both large individuals (>143 mm) and small ones (<42 mm) were present in all sampling months. The population size structure was driven by the appearance of big cohorts of individuals less than 55 mm from May to August. The appearance of small individuals in all months suggested a prolonged spawning period at other sites in this bay or sea stars growing slowly because of food shortage. An arm length is a good predictor to wet body weight for A. amurensis. The development of gonad was relative slow from May to September but rapidly reached a peak of 20.95 in October 2010, and then dropped remarkably, indicating its spawning lasted from October to November. The same phenomenon was found from March to May 2015, suggesting another spawning during March to May, which was also verified by the results of histologic analysis on ovary. The gonad index (GI) and pyloric caeca index (PCI) tended to show a negative relationship. Due to the poor food availability, the reproductive characteristics of sea star were most likely affected by the shellfish mariculture in Yantai coastal waters.

Key words: sea star, gonad index, pyloric caeca index, shellfish mariculture, Yantai


1 Introduction
Sea star Asterias spp. widely distributes in the oceans of the northern hemisphere (Byrne et al., 1997). The specie Asterias amurensis is commonly found in the northwest and northeast Pacific, especially Russia (Kashenko, 2005), Japan (Nojima et al., 1986), Korea (Paik et al., 2005), China (Liao and You, 2002), Norton Sound Alaska (Oliver et al., 1983), and Tasmania (Morrice, 1995; Byrne et al., 1997; Ross et al., 2004, 2006). In China, A. amurensis distributes in the Bohai Bay and Yellow Sea, especially in the Yanwei fishing ground, Bohai Sea, and coastal waters of Changshan Island (Zhou et al., 1996). The outbreak of A. amurensis happened several times and caused huge damages on molusca mariculture in China and Japan since 1953 (Nojima et al., 1986; Zhou and Wang, 2008). The normal density of sea star in some bays of the Far East was between 0.12 to 0.7 ind./m² (Galyshova and Pustovalova, 2009), whereas it was up to 6.1 ind./m² during outbreak (Nojima et al., 1986). The recorded outbreaks in China’s seas began in the 1960s and became more frequent and serious in recent years (Zhou and Wang, 2008). In 2006 and 2007, the outbreak occurred in coastal waters of Qingdao with an aggregated density of 300 ind./m² and caused economic losses up to 1.5 million US dollars in mariculture of scallop, abalone, and clams (Zhou and Wang, 2008). In 2008 and 2009, similar outbreaks happened in coastal waters of Yantai and Weihai.

Asterinid species have different reproductive patterns, including seasonal or continuous breeding periodicity (Carvalho and Ventura, 2002; Barker and Xu, 1991b; Byrne, 1992; Paik et al., 2005; Pastor-de-Ward et al., 2007; Mariante et al., 2010; Micael et al., 2011; Benítez-villalobos and Martinez-garcía, 2012). The differences are related to many environmental factors, including temperature, hydrodynamics, the quantity and quality of available food, and photoperiod. The seasonal changes of sea temperature affected both larval developmental duration and growth rate (Paik et al., 2005) with the tendency of quicker development in warmer water (Strathmann, 1978; Barker and Nichols, 1983). The size-age composition and reproduction cycle for A. amurensis were different from north to south by latitude, e.g., from Russi-
Asterias amurensis is a “keystone” predator and feeds on a wide variety of prey, including commercially important bivalve species, and it is responsible for huge economic losses in bivalve mariculture. Many studies have investigated its ecology, reproduction, and feeding biology (e.g., Kim, 1968; Sloan, 1980; Nojima et al., 1986; Lockhart, 1995; Ross et al., 2002, 2003; Lawrence et al., 2011). However, there are only a few studies of A. amurensis in China and mainly focused on taxonomy, distribution (Liao and You, 2002; Zhou et al., 1996) and nutrition (Hao and Li, 1998). Some studies are gonad development and reproduction, but the results were not consistent, i.e., breeding twice a year in Russia while once a year in Japan and Australia (Byrne et al., 1997; Novikova, 1978; Kim et al., 1968; Hatanaka and Kosaka, 1958). A preliminary study showed the breeding season of A. amurensis was from November to next January along the coast water of Qingdao (Zhang et al., 2014). The Bohai Sea and Yellow Sea were once the most important marine fishery grounds in China before the 1980s. However, intensive human activities (mariculture, sewage discharge, and coastal engineering) have significantly affected the coastal water environments and degraded the marine ecosystem (Tang, 2004; Wang et al., 1995; Liu et al., 2009). The Sishili Bay (SB) had a long history of mariculture since the 1950s with different species, from seaweed Laminaria japonica and Asian kelp Undaria pinnatifida in the 1950s to scallop Chlamys farreri and Argopecten irradians, and mussel Mytilus edulis in the 1990s. The farming area in SB was up to 2.450 hm\(^2\), of which 800 hm\(^2\) for scallop, 400 hm\(^2\) for mussel, and 250 hm\(^2\) for seaweed respectively (Wan, 2012; Gao et al., 2011). The culture duration for scallop lasted for one to two years, and harvested at the end of October. The shellfish as well as the inorganic carbon from mariculture would provide abundant food supply for A. amurensis, which would lead the aggregation and thus provide an ideal area to carry out the present work.

The inconsistent results of reproduction pattern and the lack of information on the size-age composition inspire us to investigate this Chinese sea star population. The aims of the present study were: (1) to obtain basic information on monthly change of sea star population size structure; (2) to speculate the spawning characteristics of A. amurensis population in the study area based on the monthly variation of gonad and pyloric caeca indices.  

2 Materials and methods

2.1 Study area and sampling stations

Individuals of A. amurensis were collected at monthly intervals from the Yuren Pier, a scallop mariculture rafts area in SB, during May to December 2010 by SCUBA diving. For getting full yearly data, we extended the investigating period from March to May 2015 again in the same area to fill the time gap in the first stage of samples collection (Fig. 1). In each collection process, we sampled sea star individuals (up to 311 individuals, arm radius (R) being 19 to 155 mm) in same depth about 5 m to minimize the sampling error. Water temperature was measured in situ using YSI (Yellow Spring Ohio, USA).

2.2 Sampling method and treatment

Individual samples were blotted dry using absorbent paper and was then wet weighed using a 0.001 g precision electric balance. The longest arm length (R\(_{\text{max}}\)) (from the center of the disc to the arm tip) was also measured using handheld calipers with the accuracy of 0.1 mm. Thirty individuals with intact body, of medium to large size class (R=55.5 to 155 mm), were selected randomly for dissection to calculated the gonad index (GI) and pyloric caeca index (PCI), respectively. Each individual was dissected by cuts extending from the center of the aboral surface towards the apex of each arm, then the gonads and caeca from each arm were removed and weighed separately. GI and PCI were calculated as wet weight of organ/total body wet weight ×100 (Grant and Tyler, 1986; Carvalho and Ventura, 2002). The mean GI and PCI were calculated for each sampling date. Based on the changes of GI against time, the stage of gonad development could be concluded.

The minimum R\(_{\text{max}}\) of sea stars presented with gonads was normally c. 55 mm (Morrice, 1995; Hatanaka and Kosaka 1958; Kim, 1968). By this, the individuals with R\(_{\text{max}}\) shorter than 55 mm were considered as small cohort (one year) and the ones with R\(_{\text{max}}\) longer than 55 mm as big size cohorts (two or three years) in the present work.

The differences of sea star size, gonad and pyloric caeca indices during eight sampling months were tested by the analysis of variance (ANOVA) and Kruskal-Wallis ANOVA analysis (Chi square) in case of heterogeneity of variance. Relationship between gonad and pyloric caeca indices was analyzed using a simple linear regression. Figures were drawn with software Matlab.

To support the GI, histological evaluation was performed on the gametogenesis and gonad maturity pattern from March to May (in the mid to late part of every month). Gonad from each individual was fixed in Bouin’s solution for at least 24 h and then transferred to 70% ethanol. The middle section of the gonad was dehydrated, embedded in paraffin and sectioned (7 μm thick). The sections were stained with haematoxylin and eosin, and then the maturity stage of gonads were examined based on the gametogenesis state and staining properties of oocytes and spermatoocytes (Kim, 1968; Byrne, 1992). Gametogenesis development stages were divided and used as evidence of gonad development, according to the results from two typical previous researches (Byrne et al., 1997, Zhang et al., 2014).

3 Results

3.1 Population size structure of A. amurensis

The body size of individuals ranged from 25 mm to 143 mm in R\(_{\text{max}}\), and small individuals (<42 mm) presented in all months. The distribution histograms of R\(_{\text{max}}\) were characterized by the al-
ternating appearance of the small (<55 mm) and big cohorts (>55 mm) in different months (Table 1, Fig. 2). From May to August, the size structure was dominated by large size cohorts and changed to small size individuals in September. In the following three months, October, November and December, the population were dominated by big cohorts again. Considering all the individuals collected in same area, the \( R_{\text{max}} \) of \( A. \ amurensis \) in eight sampling months were significantly different \( (\chi^2 = 614.04, P<0.001) \).

### Table 1. The \( R_{\text{max}} \) range charaters of \( A. \ amurensis \) collected at Yantai from May to December 2010 and March to May 2015

<table>
<thead>
<tr>
<th>Month/Year</th>
<th>( R_{\text{max}} ) range/mm</th>
<th>( n )</th>
<th>Mean</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>05/2010</td>
<td>25–100.6</td>
<td>219</td>
<td>56.8</td>
<td>±1.12</td>
</tr>
<tr>
<td>06/2010</td>
<td>41.5–134.5</td>
<td>178</td>
<td>78.2</td>
<td>±1.49</td>
</tr>
<tr>
<td>07/2010</td>
<td>38–123</td>
<td>256</td>
<td>78.1</td>
<td>±1.74</td>
</tr>
<tr>
<td>08/2010</td>
<td>41–143</td>
<td>245</td>
<td>81.3</td>
<td>±1.74</td>
</tr>
<tr>
<td>09/2010</td>
<td>28–119</td>
<td>284</td>
<td>65.7</td>
<td>±1.75</td>
</tr>
<tr>
<td>10/2010</td>
<td>33–139</td>
<td>311</td>
<td>79.3</td>
<td>±1.92</td>
</tr>
<tr>
<td>11/2010</td>
<td>38.5–126.5</td>
<td>299</td>
<td>75.5</td>
<td>±1.43</td>
</tr>
<tr>
<td>12/2010</td>
<td>29–115</td>
<td>286</td>
<td>68.3</td>
<td>±1.54</td>
</tr>
<tr>
<td>03/2015</td>
<td>41.5–120.67</td>
<td>67</td>
<td>71.2</td>
<td>±1.64</td>
</tr>
<tr>
<td>04/2015</td>
<td>33–95.7</td>
<td>123</td>
<td>59.9</td>
<td>±1.53</td>
</tr>
<tr>
<td>05/2015</td>
<td>33–116</td>
<td>47</td>
<td>71.4</td>
<td>±1.90</td>
</tr>
</tbody>
</table>

Note: \( n \) means number and SE standard error.

#### 3.2 Arm length and body wet weight

There was significant correlation between \( R_{\text{max}} \) and wet weight of \( A. \ amurensis \) (Pearson correlations, \( r=0.871, P<0.01 \)), which indicates the \( R_{\text{max}} \) was a good predictor for body wet weight (Fig. 3). The quadratic polynomial formula \((y=0.017x^2+14.285)\) can be adopted to describe the relationship between wet weight \((y)\) and arm length \((x)\).

#### 3.3 Gonad and pyloric caeca indices

The presence of gonad varied with body size and months. The smallest body size of sea stars with gonad was 49.5 mm during all the sampling months. The monthly variations of GI were significant different (ANOVA, \( F=77.81, P<0.001 \)), indicating the obvious seasonal cycles of reproduction (Fig. 4). The GI peaked in October with the value of \((20.95±6.52)\%\) (8.28–39.19), then dropped in November ((5.31±5.1)%) and December ((4.96±4.9)%), indicating a spawning event happened from October to November. This coincided well with the lower bottom water temperature (14.29°C) recorded at October 2010, though there was no significant correlation between seawater temperature and GI \((r=–0.29, P>0.01)\) (Fig. 5).

The PCI varied significantly from 3.2% to 21.7% with the average value of \((15.2±4.1)\%\) in sampling months (ANOVA, \( F=21.6, P<0.001 \)). From May to August 2010, PCI gradually increased, then decrease in September, and dropped markedly to the minimum value in October, and increased again in November 2010, suggesting the seasonal cycles of development. However, the correlation between seawater temperature and PCI was not significant \((r=0.15, P>0.01)\).

GI and PCI presented a significantly negative correlation \((r=0.85, P<0.001)\) (Fig. 3). The lowest PCI value coincided well with the maximum value GI in October, and maintained a high value from May to September, then increased markedly in November and December, which is converse to the GI development.

#### 3.4 Ovary development from March to May 2015

Individuals with gonad were mainly collected in late March and April 2015 and only one individual with gonad out of 30 individuals was found in late May. Due to indistinct results of testis histology, only the ovary maturity development was shown here (Fig. 6).

Five maturity stages of ovary development were observed during our sampling months:

- **Stage I: recovery stage**
  - The gonad wall is thick with evident two-sac structure (Fig. 6a). The gonad lumen is filled with amorphous materials and may also contain some eosinophilic cell debris and phagocytes (Fig. 6a). Nests of oogonia and many small oocytes are present in the germinial epithelium. Ovaries in this stage were mainly found in samples collected in late April and May.
  - Stage II: growing stage
  - The germinal layer is lined up with primary oocytes at various development stages (Figs 6b–c). The germinal epithelium has a tortuous outline, and extensions of the haemal layer occupy the center of the germinal folds. The haemal sinus expands to form a conspicuous layer of eosinophilic haemal fluid underlying the germinal layer. Vitellogenic oocytes are present by characteristic pear-shape. Fully grown oocytes are beginning to fill the lumen, being round and eosinophilic. Ovaries in this stage were mainly found in samples collected in mid-March.

![Fig. 2](image-url). Size frequency distribution of \( A. \ amurensis \) collected in Yantai during May 2010 to December 2010 and March 2015 to May 2015. Size classes in 4 mm intervals.
Stage III: mature stage

The fully grown oocytes are becoming large and densely-packed in the lumen (Fig. 6d). Pre- and mid-vitellogenic oocytes present in the germinal layer continuing their development. The oocytes occlude the lumen and the ovary wall becomes thin. Ovaries in this stage were mainly found in samples collected in mid-March.

Stage IV: spawning stage

The ovary wall is thin (Figs 6e–f). Fully grown oocytes are loosely packed in the lumen with more spaces due to gamete releasing. The size of these spaces and the number of large oocytes depend on the stage of spawning (early-spawning Fig. 6e, late-spawning Fig. 6f). A few pre- and mid-vitellogenic oocytes still remain in the germinal layer (Fig. 6f). Some relict oocyte debris and phagocytic tissue may be present in the lumen. Ovaries in this stage were mainly found in samples collected in mid-March.

Stage V: spent stage

The gonad wall is becoming thick with evident two-sac structure again (Fig. 6g). After the spawning, few oocytes (eggs) still presents in the ovaries, which will be resorbed and disappear later. A few pre-, early- and mid-vitellogenic oocytes remain in the germinal epithelium. Phagocytes and eosinophilic egg debris are also present in the lumen. Ovaries in this stage were mainly found in samples collected in mid-March.

4 Discussion

4.1 Population size structure

The high predatory proficiency of A. amurensis can cause serious ecological and economic impacts (Byrne et al., 1997), especially on fisheries and aquaculture due to its preference for bivalve mollusks (Sloan, 1980; Morrice, 1995). Because of the more active and stronger feeding strength by larger size individuals, the extent of this influence depend on the features of population size structure, the age composition and growth rate. From May to December 2010, the population size structure was alternately dominated by small size cohorts (<55 mm) and large size cohorts (>55 mm) due to the recruitment by more small size individuals and their growth. Normally, the cohorts were mainly composed of individuals with the body size over 19 mm in eight months. However, there were still some small individuals appeared in all months, suggesting some individuals grow more slowly because of food limitation. The changing population size structure will lead to different extent of damage to scallop mariculture during its growing season from May to October in SB. The changing size structure observed in this work could be influenced by the recruitment, growth rate and possible mortality of juveniles, similar to report from Morrice (1995).

Asterias amurensis can reach an arm length of 78 mm in approximate 12 months with an average growth rate of about 6 mm per month in coastal water of Japan (Hatanaka and Kosaka, 1958). In Tasmania, both juvenile and small adult sea stars (R=29 to 83 mm) had been growing for a minimum period of nine months with the growth rate of 3.2–9.2 mm per month (Morrice, 1995). Both the quantity and quality of available food are important factors responsible for differences in the size of sea star population (Veyers, 1949; Harrold and Pears, 1980; Lawrence and Lane, 1982; Barker and Xu, 1991a; Morrice, 1995). In the Sishili Bay, water environment and benthic assemblages have been disturbed by human activities, including culture of bivalves which affected the distribution of dissolved inorganic nitrogen (DIN) and soluble reactive phosphorus (SRP) (Wang et al., 2012); ship-
ping which affected the distribution of diatom and silico flagellate fossils (Di et al., 2013); and different pollution sources which affected modern dinoflagellate cysts (Liu et al., 2012). The considerable scale of mariculture in the Sishili Bay and its byproduct will provide abundant food supply for \textit{A. amurensis} population. These complicated factors will also affect the growth rates of sea stars and population size structure, making it hard to calculate the precise growth rate of sea star population.

4.2 Gonad Index
The maximum value of GI coincided with the lower bottom water temperature (14.29°C) recorded in October 2010, though there was no significant correlation between them. Pastor-de-Ward et al. (2007) investigated the asteroidean species \textit{Cosmasterias lurida} in Argentina, and found the GI was not significantly correlated with seawater temperature, but with photoperiod. The response to photoperiod could ensure the food supply (phytoplankton bloom) for the larvae. However, Bos et al. (2008) found increased gonad weights in the sea star \textit{Protoreaster nodosus} from March to May (spawning period), which coincided with the increasing water temperature. The timing and length of the spawning season correlates with latitude and bottom water temperature (Morrice, 1995). Nojima et al. (1986) also found the seawater temperature of spawning was keeping around 10°C; and the spawning season in the southern part was earlier than in the northern part. From north to south, the spawning season of the sea stars was different: from June to September in Russian (Kashenko, 2005); from January to May in different latitude of Japan (Hatanaka and Kosaka, 1959; Ino et al., 1955; Kim, 1968); from April to May in Korea (Paik et al., 2005), and from July to October in Tasmania (Morrice, 1995).

![Fig. 5. The seasonal change of bottom water temperatures recorded from December 2008 to December 2010 in sampling area, Yantai.](image)

![Fig. 6. Histology of \textit{Asterias amurensis} ovaries. a. Stage I, recovering ovary is lined with small oocytes; lumen is filled with flocculent material. b and c. Stage II, growing ovary is lined with pre- (PV) to late- (LV) vitellogenic oocytes. Fully grown oocytes (MV) begin to fill the lumen. d. Stage III, mature ovary with fully grown oocytes and a few pre- (PV) and early- (EV) vitellogenic oocytes. e and f. Stage IV, spawning ovary containing loosely packed oocytes with gonad wall (W) outside in early breeding season and with a few previtellogenic oocytes (PV) in late breeding season. g. Stage V, spent ovary with phagocytes (P) and reclict oocytes (R). Scale =100 μm.](image)
There was no GI increase from May to September 2010, which indicated that gametes did not accumulate in the gonads during this period. The GI peaked to 20.95% in October and markedly dropped in December, although the value of 20.95% is lower than 28.27% and 31.84% in Tasmania (Morrice, 1995). From March to May 2015, the GI also showed a markedly drop from 4.22% in March to 0.18% in April and to 0.0% in May. The development of GI is related to food availability, habitat, and reproductive strategy (Bos et al., 2011; Micael et al., 2011). Considering the changes of GI value, local bottom water temperature (14.29°C) and ovary development from March to May 2015, we confirmed that one spawning happened from October to November, and the second spawning season happened from March to May in spring with a relatively lower fecundity. Spring spawning season of *A. amurensis* was also confirmed by the complete process of ovary maturation displayed in the histologic results of ovary development. During the histologic dissection, only one individual was found with gonad out of 30 individual samples in late May, which also indicates that the spawning season was ended by May. For *A. amurensis*, male and female development is synchronized and no protogynous or protandry phenomenon was found (Veevers, 1949; Byrnes et al., 1997; Novikova, 1978; Kim et al., 1968; Hatanaka and Kosaka, 1958; Zhang et al., 2014). Therefore only the ovary development can represent the reproductive pattern of this *A. amurensis* population. The Russian researcher also found two peaks in the maturation of gonads, one in June and the other in September, with the water temperature of 17°C and 23°C, respectively (Novikova, 1978). Nevertheless, only one spawning season was observed in Japan and Korea, e.g., from April to May in Korea (Paik et al., 2005), and from January to May at around 10°C in Japan (Hatanaka and Kosaka, 1958; Ino et al., 1955; Kim, 1968). It is noteworthy that only one spawning season from October to January was observed in *A. amurensis* from Qingdao coast which was not consistent with our results (Zhang et al., 2014).

The onset of spawning in *A. amurensis* could be influenced by the combination of several environmental factors, such as food resources, fresh water influx and temperature (Morrice, 1995). The reproductive stages in asteroids should combine several parameters, such as oocyte area and diameter in female, spermatogenic columns and types of germinal cells in the lumens, as well as the presence or absence of sperm in male (Barker and Xu, 1991a, b). Based on the baseline information on monthly variation of gonad and pyloric caeca indices attained in this work, further study will need to determine whether the phenomenon found in *A. amurensis* Chinese population is intrinsic.

The body size of sea star with gonads present was c. 55 mm in arm length (Morrice, 1995), which is same to the Japanese populations in the Sendi Bay (Hatanaka and Kosaka, 1958) and the Mutsu Bay (Kim, 1968). However, the smaller size c. 46 mm in arm length reaching sexual maturity was reported in the Tokyo Bay (Ino et al., 1955). In the present study, the smallest size of sea star measured with gonad was 49.5 mm in arm length, which is similar to the previous study. 4.3 Pyloric caeca index

The pyloric caeca of sea stars has many nutritional and physiological functions, e.g., digestive enzyme production, digested product assimilation and reserve material storage (Farrand and Williams, 1988). The reserved materials can be utilized later by the gonads in the period of gametogenesis. Thus, the PCI can indicate the nutritional conditions of populations (Barker and Xu, 1991a, b). The PCI of Chinese *A. amurensis* population varied monthly from 3.2% to 21.7%, with the average value of 15.5% and the changing pattern of PCI was just opposite to GI. Our results are similar to the Japanese population of 4.0 to 23.2 with an average of 15.21±4.10 (Nojima et al., 1986). The changing pattern of PCI, however, was different from other sea star species. Keesing et al. (2011) found the PCI of *Archaster angulatus* ranged from 2.1% to 4.3%, which is much lower than *A. amurensis*. The main reason was possibly resulted from the different feeding biology of two species: *A. angulatus* is a passive deposit feeder with low nutritional diet value while *A. amurensis* is an active carnivorous feeder with high quality and nutritional diet value. The inverse correlation between the GI and PCI of *A. amurensis* could be an energy strategy for the sea star, which enables the species to maintain a high level of potential reproductive output (Scheibling, 1981; Micael et al., 2011). This distinct reciprocal relationship also displays in sea star *Acanthaster planci* (Bos et al., 2013). Previous study observed that *Sclerasterias mollis* transferred nutrient from the pyloric caeca to the gonad during the period of gametogenesis (Barker and Xu, 1991a, b). Gonadal growth requires nutrients and needs a minimum amount of energy for maintenance (Carvalho and Ventura, 2002). The quantity and quality of food availability may strongly influence the nutrient allocation in body components in asteroid which can alter the reciprocal relationship between GI and PCI (Miller and Lawrence, 1999) as well as the larval development (Basch, 1996). However, many sea stars can change their food requirement and habitat during ontogenetic development (Bos et al., 2011). In our study area, all the samples were collected inside the scallop culture zone where the food supply for *A. amurensis* was available before October each year, i.e., the harvesting season of shellfish culture. However, followed by the scarce food source from November to February next year due to the absence of shellfish culture, *A. amurensis* would have to find new food source and habitat in the study area during their ontogenetic development.

4.4 Suitability of two indices in sea star population characters

GI is an indicator of the development stage of gonads, which reflects the gonad size in relation to the body size (weight) at a particular time point (Barker and Xu, 1991a, b). However, the use of GI was controversial and some researchers considered it as an inappropriate indicator to reflect the reproductive feature or it should be used cautiously (Devlaming et al., 1982; Packard and Boardman, 1999). Meanwhile, it was also adopted by many investigations on the reproductive features of sea star (Franz, 1986; McClary and Mladenov, 1989; Ventura et al., 1997; Guzmán and Guevara, 2002; Carvalho and Ventura, 2002; Lawrence et al., 2011). Carvalho and Ventura (2002) found that the spawning started earlier than suggested by the GI according to histological analysis of gonads. However, GIs also documented a massive spawning period. The eggs released earlier were probably not fertilizable due to small egg size (Carvalho and Ventura, 2002), and then would not affect the population fertilization ratio. The aim of this work was primarily to understand the population size structure, monthly variation of gonad and pyloric caeca indices. We adopted the gonad index and physiological evaluation to investigate and predict the massive reproduction features of Chinese sea star population. The most important aspect is to clarify the population dynamics and the mechanism of outbreak of the sea stars (Nojima et al., 1986). Lockhart (1995) reported *A. amurensis* had a natural aggregated behavior, which provides the basic biological theory as the intrinsic factor for outbreak. This study provides important insights about the size structure, gonad and pyloric caeca indices of the sea star *A. amurensis* in China. However, due to the complexity of environmental factors and
food supply in study area, no satisfactory answers have been obtained up to now. Further investigation is needed on long-term and extensive surveys of this species and the changing environmental factors in relation to the global warming.

5 Conclusions

This study provides basic information to understand the life-history and reproductive strategy of Asterias amurensis, with the following conclusions:

1. The population size structure of the sea star was characterized by the alternating appearance of the small (<55 mm) and big cohorts (>55 mm) in different months. The arm length of Asterias amurensis was a very good predictor to wet body weight.

2. The reversal correlation between GI and PCI could reflect an energy strategy for the sea stars. Considering histology result of ovary, we speculated that there are two spawning periods occurred from October to November in later autumn and March to May in spring in the coastal waters of Yantai.

3. Gonad and pyloric caeca indices could be used to reflect the gonad development. However, physiological study should be added for more predictability accuracy.

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Degradation of malachite green dye by *Tenacibaculum* sp. HMG1 isolated from Pacific deep-sea sediments

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Abstract

A deep-sea bacterium from the Pacific Ocean identified as *Tenacibaculum* sp. HMG1 was found to have strong malachite green (MG) degradation activity. The MG tolerance and decolorizing activities of strain HMG1 were confirmed by bacterial growth and high-performance liquid chromatography (HPLC) analyses. Strain HMG1 was capable of removing 98.8% of the MG in cultures within 12 h and was able to grow vigorously at 20 mg/L MG. A peroxidase gene detected in the genome of strain HMG1 was found to be involved in the MG biodegradation process. The corresponding recombinant peroxidase (rPOD) demonstrated high degradative activity at 1 000 mg/L MG. Based on the common candidate intermediates, strain HMG1 was inferred to have one primary MG degradation pathway containing rPOD. In addition, five other candidate intermediates of the rPOD-MG degradative process were detected. The optimal conditions for MG degradation were determined and showed that strain HMG1 and the rPOD enzyme could maintain high bioactivity at a low temperature (20°C), variable pH values (6.0–9.0), higher salinities (100 mmol/L) and other factors, such as multiple metal ions, H₂O₂, and EDTA. MG-tolerant strain *Tenacibaculum* sp. HMG1 and its peroxidase have prospective applications as environmental amendments for MG degradation during coastal remediation.

Key words: deep-sea sediment, *Tenacibaculum mesophilum* HMG1, peroxidase, malachite green degradation characteristics


1 Introduction

Marine environments are one of the most diverse ecosystems due to their constantly variable environmental conditions. Consequently, marine bacteria develop the ability to adapt to extreme environmental conditions and become more capable of rapid adjustment in response to environmental changes and deterioration (Dash et al., 2013). Therefore, marine bacteria have the potential to be utilized for the bioremediation of recalcitrant chemicals via precipitation, volatilization, energy-dependent efflux systems, intracellular sequestration by proteins, enzymatic degradation, biofilm formation, etc. (Sakalle and Rajkumar, 2009; Vu et al., 2009; Von et al., 2002).

Dyes typically have complex aromatic molecular structures that make them more stable and more difficult to biodegrade. Global industrialization has resulted in the widespread contamination of marine environments through the constant addition of organic and inorganic wastes. Malachite Green (MG) is a synthetic triphenylmethane dye that is extensively used in the textile industry. In addition, MG was widely used in aquaculture as a parasiticide and fungicide. Although the use of MG has been banned in several countries due to its health risks, which include effects on the immune and reproductive systems as well as genotoxic and carcinogenic properties, it is still being used in many areas worldwide due to its low cost, ready availability and high efficacy (Cha et al., 2001; Srivastava et al., 2004; Zhai et al., 2007). Effluents with MG also reduce light penetration and substantially increase the biochemical oxygen demand (BOD) and chemical oxygen demand (COD), which reduce the biodiversity and capabilities of aquatic ecosystems (Novič et al., 1988; Garg et al., 2004; Darajeh et al., 2014). Moreover, the MG that remains accumulates in animal bodies and is transferred to humans through the food chain. As a consequence, various methods to remove this dye from aqueous solutions have been studied, and they can be divided into physical adsorption, chemical destruction and biological degradation methods.

Utilizing the metabolic potential of microorganisms to degrade or transform organic contaminants into less harmful substances that can be integrated into natural biogeochemical cycles is the important goal of bioremediation technology. In recent years, biodegradation methods have become appealing approaches for decomposing this recalcitrant compound. Microorganisms with the ability to degrade MG have been reported, such as *Micrococcus* (Du et al., 2013), *Saccharomyces cerevisiae* (Jadhav and Govindwar, 2006), *Pseudomonas* (Du et al., 2011; Tao et al., 2017), and *Kocuria rosea* (Parshetti et al., 2006). Desmethyl malachite green, leucomalachite green, desmethyl leucomalachite green and N, N-dimethylaniline are the common metabolic intermediates of MG biodegradation (Du et al., 2011;
Du et al., 2013; Wang et al., 2012). However, most reported microbial strains were isolated from soil and freshwater. Therefore, the property of these strains that low physiological activities in marine environments could limit capacities for the treatment of coastal MG pollution, compared with marine-derived microorganisms. Alternatively, there have been sporadic reports on the degradation of dyes by marine-derived enzymes or fungi. Torres et al. (2011) found that some marine fungal strains were capable of discoloring and adsorbing dyes after 3–4 weeks of incubation at room temperature, and Raghukumar et al. (2008) treated colored effluents with lignin-degrading enzymes derived from marine fungi.

In the present study, a marine bacterium that showed relatively high MG degradation activity was isolated from deep-sea sediments in the Pacific Ocean. The MG degradation-related enzyme peroxidase was detected, and recombinant peroxidase (rPOD) expressed in E. coli BL21 (DE3) cells was verified to rapidly degrade high concentrations of MG. In addition, the pathway and characteristics of MG degradation were analyzed to further understand the MG degradation process and the harmfulness of its products. These experimental data revealed that the strain and its enzyme have bioremediation potential for the removal of MG from aquatic solutions and marine environments.

2 Materials and methods

2.1 Samples

Samples were collected from Pacific deep-sea sediment at Site 22IX-S026-CC1109-TVMC14 (8°53′0″N, 142°59′31″E; water depth, 5 493 m) using a gravity corer. Approximately 3 cm of sediment was collected from the bottom of the gravity corer. Under sterile conditions, the surface of the sample was immediately discarded, and the remainder was stored at 4°C. The surface of the sample was again discarded after it was transported to the lab, and only the central section of the sample was subjected to further analysis.

2.2 Isolation and identification of the MG-degrading bacterial strain HMG1

The isolation of strain HMG1 for MG decolorization was conducted using the following protocol. First, 50 mL of liquid 2216E medium with 5 mg/L MG (Sangon Biotech Co., China) were mixed with 5 g of deep-sea sediment, and the mixture was incubated at room temperature until its green color faded. Next, 10 μL of the culture supernatant was inoculated into another 50 mL of fresh liquid 2216E medium with 10 mg/L MG, further enriching the domesticated MG-degrading strains. Finally, 10 μL of the supernatant from the second culture was diluted (10^−4), plated on 2216E agar, and incubated overnight at room temperature. One strain that grew on the MG screening plates was observed to have greater zones of clearing than the other strains, suggesting that it had the strongest MG degradation activity. This decolorizing strain was named HMG1, and a single colony was selected, cultivated, and identified by 16S rRNA gene sequence analysis (Weisburg et al., 1991).

2.3 Decolorization experiments with strain HMG1

The decolorization efficiency of strain HMG1 was measured using high-performance liquid chromatography (HPLC). Strain HMG1 bacterial cells were cultured in 2216E liquid medium at 30°C with shaking, and then an additional 20 mg/L of MG was added under static conditions. An aliquot of the broth was collected at different time intervals, 4 h, 8 h, 12 h, 16 h, 20 h, 24 h and 28 h, and extracted with dichloromethane (chromatographic grade, Sigma-Aldrich Co., USA) three times. The extracts were evaporated to dryness at 30°C and further dissolved in 1 mL of acetonitrile (chromatographic grade, Sigma-Aldrich Co. LLC, USA). The HPLC system (LC-20a Series, Shimadzu, Japan) was equipped with a C18 analytical column (4.6 mm×250 mm, 5 μm, LC Sciences, Japan), and measurements were performed using a mobile phase containing acetonitrile (chromatographic grade, Sigma-Aldrich Co. LLC, USA) and 0.125 mol/L ammonium (pH 4.5, Sinopharm Chemical Reagent Co., China) (80:20 (v/v)) at a flow rate of 1.0 mL/min. The detection wavelength was 622 nm, and the injection volume was 20 μL (Ren et al., 2007). The decolorization efficiency (E_d, %) was calculated by the following formula:

\[ E_d = \left( \frac{A_i - A_f}{A_i} \right) \times 100 \%
\]

where \( A_i \) is the initial peak area of MG, and \( A_f \) is the final peak area of MG. All experiments were performed in triplicate.

2.4 Identification of the peroxidase gene in the HMG1 genome using draft genome sequencing

The genome sequence of HMG1 was determined using an Illumina HiSeq2000 platform at Shanghai Majorbio Bio-Pharm Technology Co. (China). All of the reads were assembled with SOAPdenovo, and the open reading frames (ORFs) were predicted with Glimmer 3.0. The rRNA and tRNA genes were evaluated using the RNAmer and rNAscan-SE servers, respectively. The scaffolds were searched against the SWISSPROT database to identify enzymes potentially involved in MG biodegradation. A phylogenetic analysis was performed by building a Neighbor-Joining tree with the MEGA5.10 program using target ORFs and similar proteins from 15 other microbes.

2.5 Enzyme expression and purification

A peroxidase gene detected in the HMG1 genome was further cloned using primers designed according to the ORF sequence. The forward and reverse primers were 5′-AACGTCGACTAACAAATATCCTAAGGGTAGTAAACGACG-3′ and 5′-ATCCGGGCGGCCAAGATCTCAATACTCAACACCTATCTAGG-3′ and contained a SstI and NorI site, respectively. The PCR products were ligated into the pET-32a vector (Novagen, US) and transformed into E. coli BL21 (DE3) cells. Positive clones were grown in LB medium supplemented with ampicillin (100 μg/mL) and glucose (0.7%) at 37°C with vigorous shaking to an OD600 of 0.5. Expression of the peroxidase was then induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture at a final concentration of 1 mmol/L, followed by incubation at 18°C for 20 h. The cells were harvested and ultrasonicated, and rPOD was purified via nickel-affinity chromatography by elution with 200 mmol/L imidazole. The rPOD was used for the determination of MG degradation activity.

2.6 Characterization of rPOD decolorizing activity

The decolorization efficiency of MG by rPOD was determined by UV-visible analysis with a UV752N spectrophotometer (Shanghai Yok Instruments Co., China). The reaction mixture, which included 100 μL of purified rPOD, 1% H2O2, and MG (20 mg/L, 100 mg/L or 1 000 mg/L) in 20 mmol/L Tris-HCl (pH 8.0),
was incubated at 30°C, and the absorbance of the supernatant at 622 nm was measured at different time intervals, 0.5 h, 1 h, 2 h, 3 h and 4 h. The decolorization efficiency was calculated using the same formula as in the decolorization experiments, except that A and B are the initial and final absorbances, respectively.

The effect of temperature on the decolorization efficiency was determined by measuring the decolorization percentage of 100 mg/L MG in 0.5 h at 20°C, 30°C, 40°C, 50°C and 60°C. Similarly, the effect of pH was determined at 30°C in acetate-NaOH (pH 3.0–6.0), phosphate-NaOH (pH 7.0–8.0), and Tris-HCl (pH 9.0–10.0) (Zhang and Zeng, 2008; Chi et al., 2014; Han et al., 2016) buffers. The effect of H₂O₂ was determined at 30°C by adding 0%, 0.5%, 1%, 2% and 3% H₂O₂ to the reaction mixture. The effect of metal ions was determined by adding ZnSO₄, MgSO₄, MnSO₄, NiSO₄, FeSO₄, CuSO₄, Al₂(SO₄)₃, and CaSO₄ at final concentrations of 1 mmol/L to the reaction mixture. The effect of potential inhibitors was determined by adding NaCl (1 mmol/L, 10 mmol/L and 100 mmol/L), EDTA (1 mmol/L, 10 mmol/L and 100 mmol/L) and SDS (0.1% and 1%) to the reaction mixture. All experiments were performed in triplicates.

2.7 Determination of MG biodegradation intermediates by LC-MS

To determine the MG biodegradation intermediates of strain HMG1, 20 mg/L MG was added to a cell culture and incubated at 30°C for approximately 10 h. The fermentation broth was lyophilized and extracted with methanol (chromatographic grade, Sigma-Aldrich Co., USA) in the dark. To determine the MG biodegradation intermediates of rPOD, the same operation was performed, except incubation times of 15 min and 45 min were used.

All of the samples were analyzed using LC-MS (1290 LC 6490 QQQ, Agilent Technologies, USA) with an ESI ion source. The mobile phase contained ammonium (20 mmol/L, pH 4.5) and acetonitrile. The initial proportion of acetonitrile was 10%, and it reached 100% within 50 min. The injection volume was 5 μL, and the default settings were used for the other parameters.

2.8 Gene accession number

The nucleotide sequence of the 16S rRNA gene and peroxidase gene from strain HMG1 have been deposited in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) under the accession numbers KM047889 and KM047890, respectively. Genomic sequences of HMG1 have been deposited in the GenBank database under the accession number LDDQD00000000.

3 Results

3.1 Identification of strain HMG1 and characterization of its decolorization activities

Strain HMG1 had an irregular colony shape with a spreading edge and exhibited the strongest decolorizing activity on the screening medium. The 16S rDNA gene sequence showed 99% similarity with Tenacibaculum sp. MBIC11140. Examination of its decolorizing activities affirmed that strain HMG1 was capable of quickly decolorizing MG at 20 mg/L, and the decolorization rate increased nearly linearly from 0 h to 4 h (Fig. 1). At an initial concentration of 20 mg/L, after 4 h, HMG1 had degraded 94.9% of the MG. Afterwards, the MG degradation rate slowed, but incubations of HMG1 with 20 mg/L MG for 8 h, 12 h, 16 h, 20 h, 24 h and 28 h resulted in 97.3%, 98.8%, 99.2%, 99.3%, 99.4% and 99.6% MG decolorization efficiency, respectively. High concentrations of MG (100 mg/L and 1000 mg/L) substantially inhibited bacterial growth, whereas the number of colony forming units per microliter (CFU/μL) increased markedly at 20 mg/L MG. The number of CFU/μL in the control group declined after 8 h (approximately 4.73×10⁹ colonies); however, the number of CFU/μL continued to increase up to 16 h at a concentration of 20 mg/L of MG (approximately 1.06×10¹⁰ colonies).

3.2 Sequence analysis of bacterial genome and MG-degrading enzyme of MG-degrading enzymes

The draft genome of Tenacibaculum sp. HMG1 consists of 3 420 298 bases and has a G+C content of 31.55%. All of the reads were assembled into 90 contigs in 65 scaffolds. Overall, there are ten genes that encode tRNAs, one gene that encodes 5S rRNA and one gene that encodes 16S rRNA. In addition, 3 111 putative ORFs were found, giving a genes/genome percentage of 88.4%. A peroxidase gene, which, according to previous references, most likely confers MG degradation ability to HMG1 (Bhunia et al., 2001; De Souza et al., 2007), was found in the draft genome and had the highest amino acid identity (68%) to Burkholderia thailandensis E264. However, a phylogenetic tree based on amino acid sequences showed that the Tenacibaculum sp. HMG1 peroxidase, the corresponding protein of the peroxidase gene, clustered with that of Geobacillus thermodenitrificans NG80-2 and was distinct from B. thailandensis (Fig. A1).

3.3 Purification of rPOD and characterization of its MG decolorization

The peroxidase gene was cloned into the pET-32a vector for heterologous expression. The rPOD, with molecular weight of 105 kDa (Fig. 2a), showed much higher MG decolorizing ability than strain HMG1. More than 90% of MG was removed in 0.5 h at different concentrations of MG, and rPOD could degrade 96.67% of MG within 4 h at the rather high concentration of 1 000 mg/L (Fig. 2b). The optimum temperature of rPOD for MG decolorization was 30°C, which resulted in 98.73% decolorization efficiency. Moreover, high degradation activity was maintained from 20°C to 30°C, and the efficiency was still 98.65% at 20°C (Fig. 2c). rPOD exhibited high MG decolorizing activity in the wide pH range of 6.0–9.0, with an optimum pH of 8.0 (Fig. 2d). H₂O₂ greatly impacted the activity of rPOD, as an MG degradation efficiency of 99.02% was obtained with 0.5% H₂O₂, but only 33% efficiency was achieved without H₂O₂ (Fig. 2e). Small changes in decolorization efficiency showed that rPOD was not sensitive to the metal ions tested in this study (Fig. 2f). Similarly, none of the tested concentrations of EDTA (1 mmol/L, 10 mmol/L and 100 mmol/L) or NaCl (1 mmol/L, 10 mmol/L and 100 mmol/L) had a significant effect on the decolorization efficiency, which still reached approximately 98% in each case. In contrast, the decol-
orization efficiency of MG decreased rapidly with 0.1% SDS (54.8%) and 1% SDS (0%) (Fig. 2g).

3.4 MG biodegradation intermediates

Using LC-MS, three and eight intermediates were found in the MG degradation process of strain HMG1 and enzyme rPOD, respectively. The candidate products were identified according to their m/z values (Fig. A2). The three peaks detected for strain HMG1 represented desmethyl-MG (m/z 315), 4-(dimethylamino) benzophenone (m/z 226), and 4-(methylamino) benzophenone (m/z 212). In addition to the above peaks, the intermediates for rPOD also included didesmethyl-MG (m/z 302), hydroxy-MG (m/z 346), hydroxyl (didesmethyl)-MG (m/z 318), Michler’s ketone (m/z 269) and N, N-dimethylaniline (m/z 122).

3.5 Analysis of the MG biodegradation pathway

Two possible MG degradation pathways were deduced based on the detected intermediates (Fig. 3). In the first pathway, which could be involved in the degradation process of both strain HMG1 and enzyme rPOD, two demethylation reactions are catalyzed in the first step of degradation to produce didesmethyl-MG (m/z 302). Subsequently, peroxygenase seizes an electron from the central carbon of MG, and water or hydrogen peroxide serves as a hydroxylating agent (Goszczynski et al., 1994), transforming MG into a hydroxylated intermediate (m/z 318). Then, a nucleophilic reaction acts on the dye to generate 4-(dimethylamino) benzophenone (m/z 226), releasing a benzene ring-containing compound as the leaving group. Ultimately, another demethylation occurs, and 4-(methylamino) benzophenone (m/z 212) is generated for further degradation.

The intermediates detected from the rPOD degradation reactions indicate another pathway for MG degradation. In this pathway, hydroxylation first appears to produce hydroxyl-MG (m/z 346). Afterwards, the central carbon is attacked to generate Michler’s ketone (m/z 269) and 4-(dimethylamino) benzophenone (m/z 226) due to the different leaving groups. The follow-up reaction of 4-(dimethylamino) benzophenone (m/z 226) involves the above pathway. Moreover, the carbonyl of Michler’s ketone (m/z 269) would then break to generate N, N-dimethylaniline (m/z 122) for further degradation.

4 Discussion

Wastewaters discharged from different industries, such as textiles, leather tanning, paper production, aquaculture farming, food technology, and hair coloring, are usually polluted with dyes (Robinson et al., 2001). Among them, MG is most commonly used in different areas. The conventional methods for treating dyes in coastal pollution areas, including oxidation, membrane separation, activated carbon adsorption and seaweed absorption, do not show considerable efficiency or economic advantages for use in the marine environment. MG can also be transformed by zooblasts, such as Cunninghamella elegans and channel catfish muscle, but the degradation products, namely, leucomalache green (LMG) and leucogenient violet (LGV) (Chai et al., 2001; Chen and Miao, 2010), are still mutagenic and carcinogenic (Littlefield et al., 1985). One promising strategy to remediate MG pollution is the application of microbes, which are environmentally friendly, for the degradation of toxic compounds due to low running costs and nontoxic mineralized end products (Forgacs et al., 2004). In this study, Tenacibaculum sp. HMG1 was isolated from marine sediments and identified as a member of the Cytophaga-Flavobacterium-Bacteroides (CFB) group (Pinhassi et al., 1997). HMG1 is a potential candidate for the biological treatment of polluted extreme habitats because of its high adaptability and MG degrading efficiency in marine environments.

A redox reaction is essential for the degradation of MG, and various oxidases and reductases, including laccase, cytochrome oxidase P450, TMR, TpmD and peroxygenase (Zhang et al., 2009), have been reported to be responsible for triphenylmethane dye decolorization by microorganisms. To protect organisms from OH radicals, peroxygenases can remove hydrogen peroxide by transferring electrons from the donor to H$_2$O$_2$ to produce hydron (Poulos and Kraut, 1980). Peroxygenases from plants and microor-
organisms have been shown to have many applications in the degradation of biological macromolecules, such as industrial dyes (Bhunia et al., 2001; Kuhad et al., 2013; Ali et al., 2013). In this study, a peroxidase gene from strain HMG1 was identified by draft genome sequencing, and its MG decolorization activity under different conditions was further verified with recombinant enzyme expressed in *E. coli* BL21 (DE3) cells. The high decolorization activity and shared degradative intermediates demonstrated that this peroxidase likely participates in the biodegradation of MG in HMG1.

A possible MG degradation pathway was proposed based on the intermediates determined by LC-MS. The MG degradation pathway of strain HMG1 is consistent with previous investigations that showed that reductive splitting of the triphenylene rings begins with methyl group removal from the parent structure in *Shewanella decolorationis* NTOU1 (Chen et al., 2010). All possible mechanisms for the N-demethylation of N,N-dimethylamine by peroxidase were described in a previous report (Miva et al., 1983; Kedderis et al., 1983; Kedderis and Hollenberg, 1983). In addition, previous investigations have hypothesized that this process could influence the rates of the subsequent reactions (Du et al., 2013; Chen et al., 2010). An rPOD degradation pathway beginning with demethylation is consistent with the degradation process of strain HMG1, but another pathway initiated by hydroxylation instead of demethylation was also detected for the rPOD degradation process. Differences between the degradation pathways of the strain and enzyme implied that some of the intermediate products probably entered other basal metabolic pathways of the bacterium. These results also further demonstrated that the MG process occurs through degradation rather than absorption.

Interestingly, after adding MG at 20 mg/L, the number of CFU/μL of strain HMG1 increased sharply from 3.92×10^9 to 1.062×10^10, suggesting that at a certain concentration, MG could be used as a nutrient source to promote HMG1 growth. In addition, the fourfold increase in growth compared with the control group suggested the strong adaptability of strain HMG1 to an environment containing a moderate level of MG. High degradation activity (98.6%, 20 mg/L MG) at a low temperature (20°C) is also beneficial for the application of rPOD due to the typically low temperature of natural seawater (approximately 10–35°C) (Xia and Gu, 2000). rPOD was not strongly affected by environmental interference, including metal ions, EDTA (from 1 mmol/L to 100 mmol/L), NaCl (from 1 mmol/L to 100 mmol/L) and different pH values (from 6.0 to 9.0), and thus shows promise for removing MG from complex waste seawater at low temperatures, high salinities and variable pH values.

Compared with other marine fungi or freshwater bacteria, strain HMG1 and enzyme rPOD demonstrated higher MG degradation efficiency. These superior properties of strain HMG1 and enzyme rPOD in the degradation of MG indicate that they could represent good options for the remediation of MG pollution in marine environments.

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aded degradation of an azo dye—a detailed mechanistic study. BMC Biotechnology, 14: 35–47


Appendix:

**Fig. A1.** Phylogenetic tree of the peroxidase from ORF 00181. Bootstrap values of 500, calculated from 1 000 bootstrap trees, are indicated at the nodes. The numbers in the brackets are the GenBank accession numbers of the referenced amino acid sequences. The scale bar represents 0.05 amino acid substitutions per position.

**Fig. A2**

Fig. A2. LC-MS results of the probable intermediates of MG degradation. a. MG (m/z 329), b. desmethyl-MG (m/z 315), c. 4-(dimethylamino) benzophenone (m/z 226), d. 4-(methylamino) benzophenone (m/z 212), e. desmethyl-MG (m/z 315), f. didesmethyl-MG (m/z 302), g. hydroxyl-MG (m/z 346), h. hydroxyl didesmethyl-MG (m/z 318), i. Michler’s ketone (m/z 269), j. 4-(dimethylamino) benzophenone (m/z 226), k. 4-(methylamino) benzophenone (m/z 212), and l. N, N-dimethylaniline (m/z 122).
Effects of different types of nutrient effluent from shrimp ponds on the seedling growth of *Kandelia obovata*

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Abstract

Extensive shrimp ponds are located next to the landward edges of most of mangrove forests in China. A shrimp pond may influence mangroves by (1) routine effluent between pond and tide, and (2) dredging effluent from pond-dredging at least once a year. Our study consisted of two experiments to study the effects of these two effluents on the seedling growth of *Kandelia obovata*. One experiment simulated the effects of routine effluents. The other simulated four sedimentation thicknesses (0 cm, 2 cm, 4 cm, 8 cm) over mangrove soils by dredging effluent from pond-dredging, and revealed the cumulative effects of dredging effluents on *K. obovata*. At each of the three fixed salinities, i.e., 5, 15 and 25, routine effluent did not result in significant differences in each of the measured growth parameters of *K. obovata* seedlings. However, effects of dredging effluent on seedling growth of *K. obovata* were related with sedimentation thickness. Most growth parameters showed maximum values at sedimentation thickness 4 cm. The data indicated that *K. obovata* accelerated its growth under moderate sedimentation thicknesses and it was tolerant and adaptable to shrimp pond-cleaning effluent sediments up to about 8 cm in our experiment.

Key words: routine effluent, dredging effluent, shrimp pond, excessive nutrients, biomass allocation, mangroves


1 Introduction

Mangrove wetlands have been known as a low-cost and high-efficiency system for the treatment of municipal wastewater (Tam and Wong, 1997). Most mangrove wetlands are located in developing countries where anthropogenic activities are frequent and usually cause irreversible environmental degradation (Serrano-Grijalva et al., 2011). In recent years, aquaculture activities have grown fast. Food and Agriculture Organization (FAO, 2012) forecasted that by the year of 2022, world fisheries and mainly aquaculture production would provide additional 22 billion kg fish. Aquaculture especially shrimp production has rapidly developed near mangrove forests.

Since intensive shrimp culture requires high input of pelletized feed, it generate large amount of organic wastes and unassimilated food. Shrimps only make use of approximately 20% of nitrogen added to ponds as shrimp food (Briggs and Fvnge-Smith, 1994) and most part is reserved in routine effluent as exchanged water between ponds and tide, as well as dredging effluent as sludge water from ponds during dredging periods. Related study also showed that phosphorus is considered to be one of the main limitations for plant production in tropical areas (Sanchez, 2002).

Mangroves could absorb nutrients from wastewater and the wetlands are effective in removing organic matter, nitrogen, phosphorus and heavy metals (Moroyoqui-Rojo et al., 2012). Moreover, most mangrove species are highly sensitive to soil nutrient availability (Boto et al., 1985; Naidoo, 2009). Nutrient addition could promote stem elongation of dwarfed mangrove (Love-lock et al., 2006), increase branch number of *Avicennia germinans* (Whigham et al., 2009) and enhance leaf production (Feller et al., 2003a). After the sixth month, NPK addition resulted in an increasing growth of mangroves (Ribeiro et al., 2015). So we made hypotheses as follows: (1) The addition of routine effluent could promote growth of *K. obovata*; (2) Increasing discharge extent of dredging effluent (increasing sedimentation thickness above mangrove soil) could also promote growth of *K. obovata*.

During shrimp pond cleaning periods, the surface sediments in pond bottom soil are flushed by using high-pressure hydraulic giants, then the sludge water is usually discharged into coastal waters through mangrove floors. Therefore, some suspended solids from shrimp pond-dredging discharges cover over mangrove soil. These processes may also cause environmental problems including disease, environmental degradation and accumulation of black and glutinous sediments in pond bottom soil (Rosenberry, 1993; Shang et al., 1998). Numerous publications reported the adverse effects of shrimp aquaculture on water quality and biodiversity in both coastal lagoons and near-shore...
maritime habitats as well as effects of urban sewage, livestock wastewater, chemical industry wastewater and oily wastewater on mangrove forests (Boaventura et al., 1997; Duke et al., 1997; Naylor et al., 2000; Karakassis et al., 2002; Ye and Tam, 2002; Burford et al., 2003; Trott et al., 2004; Bartolini et al., 2011; Ke et al., 2011; Donato et al., 2012). However, little attention was paid to the effects of effluents of shrimp ponds including both routine and dredging effluents on mangroves. Biochemical responses of mangrove plants to inorganic pollutants and environmental conditions have been extensively reported (Takemura et al., 2000; Ye and Tam, 2002; Ye et al., 2005; Caregnato et al., 2008). Ecological responses of mangrove plants to exogenous nitrogen include the increased or decreased abundance, changes in reproduction parameters or ecosystem functions (Bartolini et al., 2009).

As for shrimp ponds, researches were confined to the quality of wastewater purification without going into details of mangrove plants. Effects of routine and dredging effluents from shrimp ponds on growth of mangrove plants were rarely reported. In case of high nutrient concentrations in sediments of shrimp ponds containing uneaten feed, dead plankton, mineral oil, airborne debris, shrimp feces, etc. (Le et al., 2005; Vaiphasa et al., 2007; Zhang et al., 2011), discharges of pond-dredging effluent would inevitably affect mangrove ecosystems. Thus, questions need to be addressed were: Did the two types of nutrient effluent make different effects on the growth of mangrove plants? Can the dredging effluent be constantly discharged through mangroves?

In China, extensive aquaculture ponds especially shrimp ponds are located next to landward edges of most mangrove forests, and Kandelia obovata (i.e., Kandelia candel in previous publications) is a predominant mangrove species which has some pioneer properties and is considered as a main reforestation species (Chen et al., 2005). This species is found in all mangrove-distributed provinces in China. In our research, shrimp farms nearby mangrove areas discharge routine and dredging effluents into coastal waters through mangrove forests. Routine effluent is frequently produced during the exchange of water between ponds and tides, while dredging effluent only appears once in a year for one pond when aquaculture section finishes. Therefore, the former is essentially shrimp pond water with nutrient levels similar to tide water but frequently disturbs mangroves, while the latter is sludge water after cleaning the pond bottom with high contents of suspended solids (SS) rich in nutrients and might have cumulative effects on mangroves due to its sedimentation on mangrove soil.

The aim of the present study was to simulate the routine exchanged water and sedimentation condition of shrimp pond-dredging discharges into mangrove forests to quantify their effects on the early development of this species. Specifically, the objectives of this study were: (1) to explore effects of these two types of effluents from shrimp aquaculture on growth of mangrove plants; (2) to explore the extent of dredging effluent discharged through mangrove floor, i.e., sedimentation thickness of shrimp pond-dredging effluent above mangrove soil.

2 Materials and methods

2.1 Materials

Mature and healthy propagules of K. obovata, which had uniform size in similar length (23.33±0.36 cm) and fresh weight (18.08±0.59 g) were collected from mangrove forest (24°24′N, 117°55′E) along the Jiulong River Estuary in Fugong Town, Longhai County, Fujian Province of China (Fig. 1). This site has tropical coastal climate of South Asia, with regular semidiurnal tide. Mangrove forests were in the high tide zone and cannot be flooded during the neap tide each month. Annual average temperature was 21°C, with lowest monthly average temperature at 13°C in January. Annual precipitation was 1 365 mm; relative humidity was 81%; and mean annual sunshine duration was 2 040.5 h. Salinity in soil was 15–21 and pH was about 7.0. Vegetation community was artificial restoration of K. obovata pure forest in banding distribution, which was about 30 m width. Intensive shrimp ponds were widely distributed in landward side. Sampling area had poor drainage facilities and effluent from shrimp ponds was always directly discharged into the surrounding mangrove areas without treatment.

At the same area, mangrove soil was collected as greenhouse pot-cultivation substrates. In August 2008, routine effluent was collected from the outlet of one shrimp pond near the mangrove forest and stored at 4°C in laboratory as treating liquids used in experiment one. In May 2009, dredging effluent (sludge water), as sedimentation materials for experiment two, was collected from the same shrimp pond through the discharge pipe for dredging effluent after flushing the pond bottom with high-pressure hydraulic giants. In addition, artificial seawater was with different salinity levels of 5, 15 and 25 for experiment one, only one salinity level of 15 for experiment two. The salt used to prepare the artificial seawater was sun-dried with natural seawater at a sea salt field in Xiamen.

Some physico-chemical characteristics such as salinity, pH, SS, TN (total-nitrogen) and TP (total-phosphorus) of routine and dredging effluents from shrimp ponds, as well as those of artificial seawater were determined (Table 1). Compared with routine effluent, dredging effluent was nutrient rich in surface sediments of shrimp pond, which contained plenty of nutrients such as nitrogen and phosphorus. The collected mangrove soil was loamy with 24.04% of particles larger than 0.02 mm, pH 7.05, water content of 42.88%, organic matter content of 2.67%, TN content of 1.32 g/kg, and TP content of 0.81 g/kg. While the shrimp pond sedimentation was clay soil, 8.88% of which had particle size larger than 0.02 mm, pH of 7.34, the ratio of water content of 49.90%, organic matter of 1.94%, TN of 1.88 g/kg, and TP of 1.48 g/kg. Polyethylene pots used for plant cultivation had 21 cm diameter and 17 cm height, and each of them had three 1 cm diameter holes at the bottom to allow rapid drainage after being irrigated.
All planted propagules successfully germinated and all seedlings were irrigated with 300 mL artificial seawater of salinity 15 every day. This experiment lasted for 224 d during which each pot was irrigated for 12 times to each of three pots with 2.5 kg mangrove soil, and forming a final steady sedimentation thickness of 8 cm above mangrove soil by dredging effluent at different salinity levels, 18 pots were divided into six treatments with 2 cm, 4 cm and 8 cm sedimentation thicknesses (ST2, ST4, ST8). K. obovata seedlings were planted in each pot with 1/3 propagule length inserted into soil. At the beginning of this experiment, propagules were allowed to germinate and grow for three months, and there were no significant differences in stem height (15.05±0.86 cm, F=0.634, P=0.778 from One Way ANOVA) and leaf number (9.03±0.15, F=1.288, P=0.331 from One Way ANOVA) of seedlings among the six treatments. The experimental design was a completely randomized block, with two fixed factors, salinity (three levels, 5, 15 and 25) and water quality (two levels, i.e., routine effluent, and artificial seawater as control). During the experimental period, for each routine effluent treated pot, 300 mL routine effluent was irrigated every two days according to the frequency of water exchange between most shrimp ponds and tide, and 300 mL artificial seawater was irrigated every day. For each control pot, we only irrigated 300 mL artificial seawater every day.

### 2.2 Routine effluent

To test growth responses of K. obovata seedlings to routine effluent at different salinity levels, 18 pots were divided into six treatments, each of which had three replicates (pots). Four K. obovata propagules were planted in each pot with 5 kg mangrove soil (1/3 propagule length inserted into soil). At the beginning of this experiment, propagules were allowed to germinate and grow for three months, and there were no significant differences in stem height (15.05±0.86 cm, F=0.634, P=0.778 from One Way ANOVA) and leaf number (9.03±0.15, F=1.288, P=0.331 from One Way ANOVA) of seedlings among the six treatments. The experimental design was a completely randomized block, with two fixed factors, salinity (three levels, 5, 15 and 25) and water quality (two levels, i.e., routine effluent, and artificial seawater as control). During the experimental period, for each routine effluent treated pot, 300 mL routine effluent was irrigated every two days according to the frequency of water exchange between most shrimp ponds and tide, and 300 mL artificial seawater was irrigated every day. For each control pot, we only irrigated 300 mL artificial seawater every day.

### 2.3 Dredging effluent

To examine growth responses of K. obovata seedlings to dredging effluent, another experiment was designed, with one fixed factor (sedimentation thickness due to dredging effluent) with four levels (treatments) of 0, 2, 4 and 8 cm due to different intensity of discharge of dredging effluent, and each treatment had three replicates (pots). Before planting propagules, substrates in pots of the four treatments were set up by (1) irrigating 800 mL artificial seawater of salinity 15 for 12 times to each of three pots with 5 kg mangrove soil as control pots (Treatment ST0); (2) irrigating 200 mL dredging effluent and 600 mL artificial seawater of salinity 15 for 12 times to each of three pots with 4.735 kg mangrove soil, and forming a final steady sedimentation thickness of 2 cm above mangrove soil by dredging effluent (Treatment ST2); (3) irrigating 400 mL dredging effluent and 400 mL artificial seawater of salinity 15 for 12 times to each of three pots with 3.75 kg mangrove soil, and forming a final steady sedimentation thickness of 4 cm above mangrove soil by dredging effluent (Treatment ST4); and (4) irrigating 800 mL dredging effluent for 12 times to each of three pots with 2.5 kg mangrove soil, and forming a final steady sedimentation thickness of 8 cm above mangrove soil by dredging effluent (Treatment ST8). Then K. obovata propagules were planted in each pot with 1/3 propagule length inserted into substrates. Before the start of this experiment, there were no significant differences in stem height (15.05±0.86 cm, F=1.793, P=0.226 from One Way ANOVA) and leaf number (6.06±0.07, F=3.000, P=0.226 from One Way ANOVA) of seedlings among the four treatments. Since propagule plantations, this experiment lasted for 224 d during which each pot was irrigated with 300 mL artificial seawater of salinity 15 every day. All planted propagules successfully germinated and all seedlings survived the whole experimental period. Stem height, stem basal diameter, branch number, leaf number, leaf water content and succulence of seedlings were determined every month but only the final data are shown in results. At the end of this experiment, all seedlings were harvested, rinsed thoroughly with deionized water, dried at 70°C to constant weight for the measurements of biomass and its partition (ratio of leaf biomass, LMR; ratio of stem biomass, SMR; ratio of root biomass, RMR; and ratio of root biomass to shoot biomass, R/S). Besides, TN and TP were analyzed for different organs of K. obovata seedlings and different matrix treatments. All of the chemical analyses were followed by the standard methods as described by Allen et al. (1974).

### 2.4 Statistical analysis

Mean and standard error (SE) of each parameter were calculated for each treatment. For experiment one, differences in stem height, stem basal diameter, leaf number and leaf fall biomass between water quality levels and among salinity levels were tested by using a parametric two-way analysis of variance (ANOVA). For experiment two, one-way ANOVA followed by Duncan’s New Multiple Range Test was used to isolate any significant difference among the four treatments. All the parameters were considered significant at P<0.05. Statistical analyses were performed by using IBM SPSS Statistics 22.

### 3 Results

#### 3.1 Effects of routine effluent on seedling growth of K. obovata

At each of the three salinity levels 5, 15 and 25, shrimp pond wastewater (routine effluent) did not result in significant differences from the control in measured parameters of K. obovata seedlings (Fig. 2). Wastewater did not have a significant impact on stem diameter while salinity effect reached significant level. Treatment under salinity 25 can suppress growth of diameter. As for leaf number, no significant differences were found at salinity of 5 and 25 between two water levels. While leaf number under wastewater treatment increased 21.86% compared with the control at salinity of 15. Salinity level affected leaf number in which it was significantly lower in salinity of 25 than value in salinity of 15 under wastewater treatment.

#### 3.2 Effects of dredging effluent on seedling growth of K. obovata

**3.2.1 Growth status**

Similar performance due to sedimentation of dredging effluent from shrimp ponds was found for the morphological parameters including stem height, stem diameter, branch number and leaf number of K. obovata seedlings after 224 d of experiment (Fig. 3). Compared with the control (ST0), the three treatments with 2 cm, 4 cm and 8 cm sedimentation thicknesses (ST2, ST4 and ST8) had higher or similar values of all of these parameters. Sedimentation thickness of dredging effluent had significant effects on stem height, stem basal diameter, branch number, leaf number and leaf water content.
impacts on stem diameter ($F=9.762$, $P=0.005$).

The tendency of biomass parameters under sedimentation thickness of dredging effluent was similar to that of morphologic- al parameters (Fig. 4). Maximum values were detected in ST2 or ST4. Significantly higher values of leaf biomass were occurred in treatments ST2 and ST4 than those in ST0 ($F=5.052$, $P=0.030$). However, this enhancement was weakened when dredging efflu- ent further sedimented to 8 cm (ST8).

Accordingly, we calculated parameters of biomass partition (Table 2). LMR and SMR were favorable under ST2 or ST4 and their lowest values were found in ST0 or ST8. On the contrary, RMR and R/S were lowest under ST4 and highest under ST0.

Sedimentation of dredging effluent had significant effects on leaf water content ($F=4.325$, $P=0.043$) (Fig. 5). For all of the four treatments, the mean leaf water content varied within 72.88%–75.95%, and the mean values of leaf succulence were 3.42–3.91 g/dm$^2$. Both parameters had similar change patterns with sedimentation thickness, and increased when the sedimentation thickness increased from 0 to 4 cm, except for the values of ST8 which were lower than ST4. Besides, we measured soil conductivity at the end of our experiment. They were 4.52, 4.73, 5.67 and 6.57 mS/cm under treatment ST0, ST2, ST4 and ST8, respect-
Table 2. Effect of simulated sedimentation thickness on biomass allocation of *K. obovata*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LMR</th>
<th>SMR</th>
<th>RMR</th>
<th>R/S</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST0</td>
<td>0.32±0.01*</td>
<td>0.33±0.01*</td>
<td>0.35±0.00*</td>
<td>0.54±0.03*</td>
</tr>
<tr>
<td>ST2</td>
<td>0.35±0.01*</td>
<td>0.34±0.02*</td>
<td>0.31±0.03*</td>
<td>0.45±0.07*</td>
</tr>
<tr>
<td>ST4</td>
<td>0.38±0.01*</td>
<td>0.33±0.01*</td>
<td>0.29±0.00*</td>
<td>0.41±0.01*</td>
</tr>
<tr>
<td>ST8</td>
<td>0.36±0.01*</td>
<td>0.32±0.02*</td>
<td>0.31±0.03*</td>
<td>0.46±0.05*</td>
</tr>
</tbody>
</table>

Note: Means and SE (standard error) are shown. SEs with different letters in each treatment indicates significant difference at *P*<0.05 according to one-way ANOVA test. LMR, SMR, RMR and R/S represent ratios of leaf biomass, stem biomass, root biomass and root/shoot, respectively. ST0, ST2, ST4 and ST8 represent simulated sedimentation thicknesses of 0 cm (the control), 2 cm, 4 cm and 8 cm, respectively.

Fig. 4. Effects of simulated cumulative sedimentation thicknesses on biomass of *K. obovata*. Mean and SE (standard error) of three replicates are shown, straight line with different letters in each treatment indicates significant difference at *P*<0.05 according to one-way ANOVA test. ST0, ST2, ST4 and ST8 represent cumulative sedimentation thicknesses of 0 cm (the control), 2 cm, 4 cm and 8 cm, respectively.

Fig. 5. Effects of simulated cumulative sedimentation thicknesses on leaf water content and succulence of *K. obovata*. Mean and SE (standard error) of three replicates are shown, straight line with different letters in each treatment indicates significant difference at *P*<0.05 according to one-way ANOVA test. ST0, ST2, ST4 and ST8 represent cumulative sedimentation thicknesses of 0 cm (the control), 2 cm, 4 cm and 8 cm, respectively.

3.2.2 Nutrition status

Table 3 showed nutrient content of different matrix treatments and changes of nitrogen and phosphorus content in different organs of *K. obovata* at the end. Dredging effluent significantly affected MN (nitrogen content in matrix) and MP (phosphorus content in matrix) under our four treatments (MN: *F*=12.693, *P*=0.002; MP: *F*=7.828, *P*=0.009). Both two indexes increased with adding of sludge. Nitrogen content in different or-
gan reached top in ST4 or ST2. Phosphorus content of root in ST4 was significantly higher than ST0 (F=7.736, P=0.009).

4 Discussion

In natural conditions, mangrove habitat is lacking of nutrients (Tomlinson, 1986; Wang et al., 2003). Many studies showed that most mangrove plants were sensitive to changes of nutrients (Boto et al., 1985; Feller, 1995; Yates et al., 2002; Feller et al., 2003b; Naidoo, 2006). Besides, adaptation to salinity levels vary by mangrove species (Downton, 1982; Clough, 1984; Kao et al., 2001) and it was reported that an increase in NaCl from 85 to 430 mmol/L significantly reduced the growth of seedlings of K. candel (Kao et al., 2001). However, routine effluent from shrimp ponds in our study did not result in predicted significant differences in seedling growth of K. obovata at each of the designed salinities (5, 15 and 25), even though the experiment lasted for 14 months, longer than most of other experiments on mangrove seedlings. It is different from discharge of livestock wastewater with NH$_4^+$-N and PO$_4^{3-}$-P contents of 36.1 and 53.7 mg/L which significantly enhanced seedling growth of K. candel (i.e., K. obovata) collected from Wong Chuk Wan mangrove swamp of Hong Kong after 144 d of treatment in greenhouse pot-cultivation systems (Ye and Tam, 2002). The reason might be that nutrient levels of routine effluent in our present study were not sufficient enough to significantly affect mangroves. For example, TN contents of routine effluent were 4.72-5.92 mg/L, at the same magnitude order as DIN contents (1.553-1.904 mg/L) of tide water at the Jiulong River Estuary during tide flooding periods in July 2012 (Qu and Ye, 2013), indicating that routine effluent had nutrient levels similar to waters adjacent to mangrove forests. Since influence of shrimp pond routine effluent is a long-term process and mangrove nature reserve in the Jiulong River Estuary has appeared to degrade in varying degree, we recommend strengthening the management to avoid discharging of routine effluent without any effective purification.

For mangrove species, because of low concentration of nitrogen and phosphorus in matrix (Boto and Wellington, 1983), nutrient content in habitat had the most direct impact on nutrient elements in mangroves. Sediments were rich in nitrogen and phosphorus when clearing shrimp ponds (Burford and Lorenzen, 2004; Hossain et al., 2009). At the end of our experiment, the maximum values of TN and TP content were mostly detected in ST4 and ST8, and discharge of moderate amount of dredging effluent can promote growth of mangrove plants theoretically. Several studies have shown mangrove wetlands were good for TN and TP removal efficiency (Sansanayuth et al., 1996). Boto and Wellington (1983) reported that adding nitrogen and phosphorus in mangrove communities would promote plant growth and lead to addition of nutrient content in plants. However, there was no significant difference in leaf nitrogen content among our four groups, it followed an order of ST4 > ST2 > ST0 > ST8. Probably the result was consistent with previous studies, excessive phosphorus fertilization may cause plant lodging, undesirable delayed senescence at later stages of growth and deterioration of grain quality (Drinkwater and Snapp, 2007; Subedi et al., 2007). Excessive phosphorus fertilizers caused environmental pollution and increased production cost (Li et al., 2016). In our study, maximum values of nitrogen and phosphorus content in leaf, stem and root of K. obovata. ST0, ST2, ST4 and ST8 represent simulated sedimentation thicknesses of 0 cm (the control); 2 cm, 4 cm and 8 cm, respectively.

### Table 3. Nitrogen and phosphorus content in different matrix treatments and K. obovata organs at the end

<table>
<thead>
<tr>
<th>Item/kg $^{-1}$</th>
<th>ST0</th>
<th>ST2</th>
<th>ST4</th>
<th>ST8</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN</td>
<td>1.36±0.03 $^a$</td>
<td>1.39±0.04 $^a$</td>
<td>1.41±0.04 $^a$</td>
<td>1.61±0.02 $^b$</td>
</tr>
<tr>
<td>MP</td>
<td>0.77±0.02 $^a$</td>
<td>0.83±0.02 $^a$</td>
<td>0.87±0.04 $^b$</td>
<td>0.96±0.04 $^b$</td>
</tr>
<tr>
<td>LN</td>
<td>13.71±0.06 $^a$</td>
<td>13.73±1.82 $^a$</td>
<td>14.04±1.61 $^a$</td>
<td>13.17±1.86 $^a$</td>
</tr>
<tr>
<td>SN</td>
<td>5.47±0.95 $^a$</td>
<td>6.19±0.50 $^a$</td>
<td>9.55±1.96 $^a$</td>
<td>7.01±0.19 $^a$</td>
</tr>
<tr>
<td>RN</td>
<td>5.15±0.55 $^a$</td>
<td>5.97±0.14 $^a$</td>
<td>5.91±0.78 $^a$</td>
<td>5.78±0.68 $^a$</td>
</tr>
<tr>
<td>LP</td>
<td>2.47±0.25 $^a$</td>
<td>2.56±0.22 $^a$</td>
<td>2.49±0.17 $^a$</td>
<td>2.61±0.13 $^a$</td>
</tr>
<tr>
<td>SP</td>
<td>1.92±0.03 $^a$</td>
<td>2.09±0.14 $^a$</td>
<td>2.49±0.18 $^a$</td>
<td>2.36±0.15 $^a$</td>
</tr>
<tr>
<td>RP</td>
<td>4.43±0.03 $^a$</td>
<td>4.54±0.30 $^a$</td>
<td>5.71±0.39 $^a$</td>
<td>5.85±0.20 $^a$</td>
</tr>
</tbody>
</table>

Note: Means and SE (standard error) are shown. SEs with different letters in each treatment indicate significant difference at P<0.05 according to one-way ANOVA test. MN and MP represent nitrogen and phosphorus content in matrix; LN, SN and RN represent nitrogen content in leaf, stem and root of K. obovata; LP, SP and RP represent phosphorus content in leaf, stem and root of K. obovata. ST0, ST2, ST4 and ST8 represent simulated sedimentation thicknesses of 0 cm (the control); 2 cm, 4 cm and 8 cm, respectively.

us in mangrove communities would promote plant growth and lead to addition of nutrient content in plants. However, there was no significant difference in leaf nitrogen content among our four groups, it followed an order of ST4 > ST2 > ST0 > ST8. Probably like previous studies, excessive phosphorus fertilization may cause plant lodging, undesirable delayed senescence at later stages of growth and deterioration of grain quality (Drinkwater and Snapp, 2007; Subedi et al., 2007). Excessive phosphorus fertilizers caused environmental pollution and increased production cost (Li et al., 2016). In our study, maximum values of nitrogen and phosphorus content in leaf, stem and root were mostly detected under small amount of dredging effluent (2 cm and 4 cm), which indicated suitable amount of sludge under 8 cm in our study was beneficial to nutrition absorption. This means utilization of dredging effluent from shrimp pond has a threshold. Accumulating sedimentation above or below the threshold in environment is not conducive to absorption of mangroves.

Unlike routine effluent, dredging effluent had high loads of nutrients such as nitrogen and phosphorus which chronically cumulated into shrimp pond sediments mainly as shrimp feces, unassimilated food, and residues (Páez-Osuna, 2001; Trott et al., 2004; Anh et al., 2010). Nutrient and particulate matter exported from shrimp ponds have been determined in some experiments (Xie et al., 2004). Study in South China reported that shrimp pond sediment wash-out resulted in TN loading of 7.204 g/(m$^2$·a), and TP loading of 35.386 g/(m$^2$·a), respectively. 100-1 000 times higher than the loading from tidal water exchange (Wu et al., 2014). In our experiment, dredging effluent had TN of 377.24 mg/L and TP of 286.44 mg/L, which were 79.92–63.72 times and 215.37–166.53 times of those in routine effluent. Among four sedimentation treatments (0 cm, 2 cm, 4 cm and 8 cm), ST4 had the highest values for almost all measured morphological parameters. That is, enhancement of dredging effluent to seedling growth of K. obovata was more obvious under moderate retention quantity of dredging effluent into mangrove forests and this impact would decrease with the further sedimentation of dredging effluent. Our result was consistent with a study of effects of residual sludge on growth parameters of Acacia auriculiformis: study recorded that the concentrations of nitrogen and phosphorus were highest in T4 (soil + residual sludge = 1:1) but the growth and biomass of seedlings were the maximum in T5 (soil + residual sludge = 2:1) (Hossain et al., 2009). During our experiment, each dredging effluent irrigation treatment especially ST4 group was leafy and had stout branches. It is different from the control group which branched less and had leaves in light green. This phenomenon also indicated that addition of dredging effluent with nutrition could promote the growth of K. obovata in a
certain extent, in contrast to lacking of nutrition in control group. It is an ecological adaptation when plants faced mineral element variation. Changing of nutrient supply in environment would change plants biomass and could lead to changing of nutrient allocation proportion in different organs (Ibrahim et al., 1998). After 224 d of culturing in our experiment, leaf biomass under treatment was significantly higher than the control and the maximal leaf, stem and total biomass was observed under ST4. This result was consistent with previous report that plant biomass in wetlands would increase with applying of nitrogen (Mitsch et al., 1994). However, our further sedimentation over ST4 of dredging effluent resulted in low biomass and biomass of ST8 was closest to ST0. It showed biomass of K. obovata increased following addition of shrimp pond dredging effluent rich in nitrogen and phosphorus, but slowed down after reaching a certain amount of irrigation. It is similar to the results from Chen et al. (1995) that K. obovata seedlings treated with natural municipal wastewater had higher biomass than those in concentrated wastewater which contained five and ten times of the nutrients. Increases in biomass and shifts of biomass from roots to shoots are well-documented responses of mangroves to soil nitrogen and phosphorus enrichment (Linder and Book, 1984; Naidoo, 2009). Similarly, nitrogen enrichment increased the biomass of all aboveground plant parts of Cyperus iria (Awan et al., 2015). In terms of biomass partition, K. obovata seedlings in our study had the highest LMR and lowest RMR at ST4, which means K. obovata allocated more biomass to the ground part especially to leaves. For ST0 lacking of nutrition, plants allocated more biomass to roots, which may be beneficial to absorption of mineral elements. Our study validated the optimal allocation theory that plants would allocate more biomass in order to increase the restrictive function of organ or part of the resource utilization (Bloom et al., 1985). Study also showed that in order to assimilate adequate nutrients, roots prolifercally grew into substrates with low nutrient levels, and plants take advantage of nutrient patches by increasing total root length and fine root production (Blair and Perfecto, 2001; Hodge, 2004).

Leaf water content is an indirect parameter reflecting plant growth, and high values were related with rapid metabolic and growth rates of K. obovata seedlings treated with wastewater (Chen et al., 1995; Ye et al., 2003). Severe water stress reduced leaf area and, consequently, reduced photosynthesis and affected metabolism, resulting in stunted growth (Lisar et al., 2012). Our study also reflected that addition of dredging effluent promoted metabolism of K. obovata seedlings in some degree. Leaf water content of K. obovata seedlings significantly increased due to sedimentation of dredging effluent at ST4 from shrimp ponds, which enhanced their growth. Soil conductivity was 4.52, 4.73, 5.67 and 6.57 mS/cm, increasing with sedimentation thicknesses, which had positive correlation to salinity at the end of our experiment. Previous studies showed that succulent leaves can store large amounts of water to reduce salt concentration and increases in leaf succulence are one of the indicators of halophytes to high salinity stress (Jennings, 1968; Short and Colmer, 1999). In the present study, sedimentation of dredging effluent increased leaf succulence of K. obovata seedlings and level of leaf succulence followed the order of ST4 > ST2 > ST8 > ST0. This maybe because increasing ion concentration in the matrix of dredging effluent influenced water absorption and caused osmotic stress. From this index, increasing succulent degree under our treatment enhanced adaptability to salt stress from dredging effluent. However, the adaptability reduced when filling thickness reaches 8 cm, indicating high sedimentation (ST8) resulted in negative effect on seedling growth compared with the moderate sedimentation (ST4) and it was not conducive to long-term growth of K. obovata under environment of this substrate.

Comprehensively, discharge of shrimp pond effluent especially dredging effluent is not suitable for the growth of K. obovata in the long run. The mangrove soil we collected was loamy with 24.04% of particles larger than 0.02 mm while shrimp pond sedimentation was clay soil, 8.88% of which particle size larger than 0.02 mm. This particle size factor may influence growth of plants since research showed that growth of mangrove had positive correlation with percentage of sediment particle size greater than 0.02 mm (De Lange and De Lange, 1994). Moreover, physical and chemical properties of mangrove soil will change after sewage irrigation of shrimp ponds, such as low permeability (Yap, 2000), which may make it difficult for reforestation. Study in Pak Phanang, Thailand, confirmed that the excess sediments discharged from nearby shrimp ponds reduced mangrove growth rates and increased mortality rates. Besides, comparison between four dominant mangrove species revealed that different mangrove species could tolerate different sedimentation depth. Avicennia marina could tolerate sedimentation rates of >6 cm/a (Vaiphas et al., 2007). Since shrimp pond sediment is clay soil and soil particle composition is an important ecological factor affecting other physio-chemical and biological characteristics of soil, further cumulative effect may be not suitable for growth and evolution of mangrove plants. In our experiment, addition of routine effluent hardly promotes growth of K. obovata and increasing discharge extent of dredging effluent (increasing sedimentation thickness above mangrove soil) could not always promote growth of K. obovata, with growth conditions in ST8 slightly higher or close to ST0. We infer that K. obovata could tolerate sedimentation rates of <8 cm/a. Thus, we advise controlling discharge of shrimp pond effluent, otherwise, it will eventually affect the function of the mangrove ecosystem.

5 Conclusions

Comprehensively, there was no expected promotion effect to growth of K. obovata from the two types of nutrient effluents. Although discharge of dredging effluent at a moderate strength (sedimentation thickness under 8 cm over mangrove soil) would enhance K. obovata growth, further dredging effluent should be prohibited in the study site because of the possible harmful substances in dredging effluent in addition of nutrients. Besides, shrimp pond sediment was clay, and changes in matrix characteristics may influence growth of mangroves. Since mangrove ecosystems are endangered in China, enrichment of shrimp pond sediments into mangrove system must be controlled. Management should be enforced in order to protect both mangroves and aquaculture development. Moreover, our study was based on laboratory culture while other effects may have in field condition. Further studies are needed to investigate the long-term and harmful substances in shrimp farm effluent.

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References


