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NMR relaxometry as a potential non-invasive routine sensor for characterization of phenotype in *Crassostrea gigas*

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Abstract

MR imaging is the most appropriate non-invasive technique for quantifying the growth of somatic and gonad tissues and to determine sex in the Pacific oyster, *Crassostrea gigas*. However, this technique is too costly for field studies where oysters are used as bioindicators of environmental quality or to be applied routinely in hatcheries. We have tested the ability of low Nuclear Magnetic Resonance relaxometry, a much less expensive technique, to obtain phenotype parameters that can be used to monitor the physiological state of oysters. NMR measurements were carried out at three different periods using a low field spectrometer equipped with a 50 mm diameter probe to investigate 60 oysters in their first year of maturity, which were then dissected to measure internal shell cavity volume and dry flesh weight and to determine sex and gonad development. The NMR results showed that it was possible to determine both internal shell cavity volume and dry flesh weight in less than one minute with very high determination R^2 coefficients (0.95 and 0.94, respectively). The results showed also that it was possible to identify sex and gonad development, with success rates of 93% and 83%, respectively. For oysters with dry weight above 0.7g, the success rate in identifying sex was 100%. Further studies are required

27 to design an NMR probe that is appropriate for larger oysters and to improve sex discrimination
28 and prediction of gonad development with larger study groups.

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31 *Keywords:* Phenotype characterization, *Crassostrea gigas*, NMR, growth, Sex identification

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1. Introduction

With an annual production of above 117,500 metric tons in 2006, production of the Pacific oyster *Crassostrea gigas* is economically important in French aquaculture. However, the ecology and physiology of this bivalve are not fully understood, and consequently monitoring of its growth and reproduction, both in the field and in hatcheries, is still based on empirical factors. Investigation of soft tissues in marine mollusks, especially in marine bivalves, classically relies on destructive methods, since a hermetic shell protects the animal. For example, anatomical structures are generally studied after opening and dissection by means of histological sections, with a resolution of 4-5 μm (Chavez-Villalba et al., 2002; Didri et al., 2007). The evolution of gametogenesis can be quantitatively assessed with the help of image analysis (Chavez-Villalba et al., 2003; Fabioux et al., 2005), and sex may be determined after anesthesia using magnesium chloride and tissue sampling using needles (Namba et al., 1995). However, analysis of physiological and biochemical changes requiring large volume tissue samples necessarily involves the sacrifice of numerous specimens and the preparation and analysis of several samples. These standard techniques provide valuable information on marine mollusk biology but have two main disadvantages which are limitations for many studies: these methods are very time consuming and they are necessarily destructive. Non-invasive and quantitative procedures have therefore been developed, and after preliminary trials (Pouvreau et al., 2006) Nuclear Magnetic Resonance (NMR) imaging (MRI) has proved promising.

Non-invasive characterization of gonad maturation and determination of the sex of Pacific oysters by Magnetic Resonance Imaging (MRI) has already been successfully tested using longitudinal relaxation T1-weighted MRI sequences (Davenel et al., 2006). MRI is the most appropriate technique for quantification of the growth of somatic and gonad tissues and to determine sex. However, this technique is too costly for field studies and particularly for routine use in hatcheries. Preliminary results (Davenel et al., 2006) of the characterization of various

oyster tissue samples by NMR relaxometry, a much less expensive technique, showed that the T1 relaxation times of gonad and muscle were shorter than those of the heart, other viscera and gills which are bathed in seawater, itself characterized by very high T1 and T2 relaxation time values (more than 1600ms). These experiments ascertained that the NMR technique was able to differentiate the ovaries (T1 of about 207 ± 21 ms) from the testes (T1 of about 456 ± 50 ms) and muscles (T1 of about 461 ± 13 ms). However, the NMR technique did not make it possible to differentiate the male gonad and muscle on the basis of the T1 value alone. We report here the ability of NMR to obtain phenotype parameters that can be used to monitor the physiological state of entire oysters in their shells by multivariate data analysis techniques on NMR transverse T2 relaxation data weighted by longitudinal T1 relaxation using different relaxation delays.

2. Materials and methods

2.1 Origin of animals and preparation

NMR measurements were carried out at three different periods (May 7, June 4 and June 29 2007) on 31 diploid (2N) and 25 triploid (3N) *Crassostrea gigas* oysters in their first year of maturity to limit their size to the 52mm diameter of the NMR probe. They were bred by the IFREMER Shellfish Laboratory located in Argenton near Brest (Brittany, France) and brought to Cemagref in Rennes (Brittany, France) for NMR investigations without any specific anesthetization procedure (distance 250 km). Before NMR measurements, animals were soaked in sea water to expel any air bubbles. Sea water was kept at room temperature with the addition of phytoplankton to facilitate the opening of oysters.

After NMR measurements, each oyster was measured and weighed using a standard method. First, individual total mass (*i.e.* shell plus tissue), was weighed to the nearest 0.1g on an electronic Sartorius balance (TW). Oysters were then opened and the flesh was drained for 15 min on absorbent paper and weighed using an electronic Sartorius balance to the nearest 0.001g to determine fresh flesh weight (FW). The difference between total weight (TW) and flesh weight (FW) provides an estimate of the internal shell cavity (ISC) expressed as mass unit (g).

Gonad development (GD) was visually estimated at four qualitative stages from stage 0 (no visible gonad tissues) to stage 3 (well developed gonad tissues). A biopsy was then taken from each gonad and analyzed under a light microscope to determine sex. The presence of spermatozoa (2-3 μm) or oocytes (30-50 μm) in the sample indicated if oysters were male (M) or female (F), respectively. Triploid oysters that were asexual were annotated 3N. Finally dry flesh weight (DW) was measured after a 72-hour freeze-drying cycle.

2.2 NMR measurements

NMR measurements were performed at Cemagref (Rennes, Brittany, France) with an OXFORD MQA 6005 spectrometer operating at 0.12T (5MHz) equipped with a 52mm vertical diameter probe which allowed investigation of oysters in their first year of maturity (< 45 g total weight). NMR data comprised the intensities of echoes originating from an NMR pulse sequence that acquired two Carr-Purcell-Meiboom-Gill (CPMG) spin echo trains with different relaxation delay (RD) times between each signal accumulation reflecting combined T1 and T2 relaxation times (Table 1). The 15000ms RD time was chosen to obtain the full T2 relaxation signal of sea water in the internal shell cavity without any T1 weighting. The 400ms RD time corresponded to the T1-weighting parameter used in previous MRI experimentations (Davenel et al., 2006) to obtain the best contrast between testes and ovaries. Low-field NMR measures the spin-spin (T2) and spin-lattice (T1) relaxation of hydrogen nuclei (protons) in mobile or less mobile molecules.

2.3 Statistical analysis

Ideally, after data treatment based on Levenberg-Marquardt or maximum entropy decomposition, low field NMR measurements lead to a distribution of spin-spin relaxation times with peaks corresponding to resonance states of hydrogen nuclei that may be related to specific classes of molecules in the sample, each class characterized by its state (liquid/solid), its diffusion rate or its type of bonding or interaction with other molecules. However, if the rate of chemical exchange of protons between different classes is faster than the time scale of the NMR experiment, two or more classes may be indistinguishable from each other and the exact number

and types of classes cannot be measured. We therefore chose to use other approaches based on chemometric techniques to extract information from the NMR CPMG relaxation curves.

Determination of quantitative phenotype characteristics (ISC, FW and DW).

Because the first echo intensity of the non-T1-Weighted CPMG sequence with long RD (15000ms) is proportional to the total proton density of the sample, we tested the simple linear relationship between ISC and the intensity of this variable, and also the intensity of the first echo of the T1-Weighted CPMG sequence with shorter RD value (400ms). Protons in the flesh were water protons interacting with macromolecules or were constituents of lipids and their T2 relaxation parameters were shorter than sea water protons. Consequently we tested the linear relationship between flesh weight (FW) or dry weight (DW) and a combination of the intensity of the first echo and the intensity of a longer echo time, strongly weighted by the transverse relaxation of the sea water protons.

The traditional univariate linear and multiple regression methods are of limited value to handle the large amount of co-linear data points acquired by CPMG sequences. Multivariate data analysis (chemometric) techniques include algorithms that can handle large co-linear data structures. In this context, the main advantage of chemometric tools is that they are able to deal with spectral or relaxation information, such as NIR and NMR (multivariate co-linear data) by reducing data to a few latent factors (LF). Correlations between quantitative phenotype and NMR data were evaluated using the partial least squares (PLS) predictive regression method on relaxation data originating from the CPMG sequence for each RD value (CPMG-400, CPMG-15000) separately and in combination with NMR data (CPMG-400-15000).

Determination of qualitative phenotype characteristics (SEX, GD).

First non-supervised principal component analysis (PCA) was performed to reduce the dimensions of the original CPMG data matrices retaining the maximum amount of variability. This provided a new set of variables (principal components, PCs) which facilitated the discovery of patterns hidden in the dataset. A stepwise linear discriminant analysis (LDA), a supervised

method used for classification purposes, was then performed on PC variables. LDA renders a number of orthogonal linear discriminant functions equal to the number of classes minus one. This method maximizes variance between categories and minimizes variance within classes. Three sex classes were defined: male (M), female (F) and triploid or asexual oysters (3N), and the four qualitative stages described above were used for classification according to gonad development of diploid oysters. Stepwise PLS and LDA procedures were combined with the leave-one-out cross-validation procedure to seek subsets of synthetic LF or PC variables that were the most useful to evaluate predictive errors (RMSEP) of quantitative phenotype characteristics or to discriminate between classes and estimate the clearly classified percentages of qualitative characters. All of the chemometrics discussed were performed using the statistical package R (R Foundation for Statistical Computing, Vienna, 2006: www.R-project.org).

3. Results

3.1 Determination of quantitative phenotype characteristics (ISC, FW and DW).

In terms of the relationships between the quantitative phenotype characteristics themselves, measured by the standard destructive methods, it should be noted that fresh weight (FW) and dry weight (DW) were highly correlated (R^2 : 0.94), demonstrating the good repeatability of the method used to measure fresh weight. Internal shell cavity (ISC) volume was moderately correlated with FW and DW (R^2 : 0.56).

Simple univariate correlation between internal cavity volume (ISC) and the intensity of the first echo (echo1) of the CPMG-15000 relaxation curve showed that it was possible to determine ISC with a very high correlation coefficient (R^2 of 0.95). In the range 4.7g to 16g, ISC was predicted with a standard error of prediction of 0.62g (Table 2).

To eliminate the influence of the sea water protons on the NMR signal, slightly more sophisticated relationships were necessary to predict FW and DW. The results showed that the best combination was to subtract the intensity of a spin echo strongly affected by sea water protons from the first spin echo of the relaxation curve. All the possible combinations were tested. After optimization, the value resulting from the subtraction of the intensity of the echo at 64 ms from the beginning of the relaxation curve from the first echo of the CPMG-400 data appeared to be the best correlated with DW and FW, with correlation coefficients of 0.94 and 0.91, respectively. In the range 0.22g to 2.10g, DW was predicted with a standard error of prediction of less than 0.1g (Table 2, figure 1). FW was predicted with a standard error of prediction of less than 0.54g in the range 1.28g to 8.90g (Table 2).

Several combinations of latent variables resulting from the PLS procedure showed good correlations but did not provide a very significant improvement in the relationships with the different quantitative phenotype characteristics such as DW and FW despite the combinations of 5 or 6 latent variables (Table 3). However, the combination of only two latent variables resulting from the CPMG-400 curves alone provided a potentially acceptable prediction of ISC (R^2 : 0.93; RMSEP: 0.78g).

3.2 *Determination of qualitative phenotype characteristics (SEX, GD).*

Stepwise linear discriminant analysis (LDA) of each CPMG curve result separately showed that SEX could be classified with a success rate of 59% and 71% with CPMG-400 and CPMG-15000, respectively. Based on a combination of the results from both CPMG curves, LDA showed a very significant increase in the success rate (to 93%) to predict SEX (Table 4). Discriminant function 1, explaining 78% of the variance, could be interpreted as well correlated with ploidy, and discriminant function 2 clearly separated 2N males and females (Figure 2).

Stepwise linear discriminant analysis based on the combination of data from both CPMG curves showed that the potential of NMR relaxometry to discriminate gonad development was good, with 84% of 2N oysters correctly classified (Table 5).

4. Discussion

These preliminary results show that the low field NMR relaxometry technique is a potentially non-invasive routine sensor for phenotype characterization in *Crassostrea gigas*. The technique provides access to two important numerical representations of the quality of soft bivalve tissue: the weight of the soft tissue and the meat condition index based on the percentage of the internal shell volume occupied by soft body tissue. The most promising result was the estimation of the fresh or dried soft tissue weight by simple bilinear regression based on the CPMG-400 curve, obtained in less than a minute. Using 400 ms RD time, the weight of the very mobile hydrogen atoms (protons) in the CPMG-400 relaxation signal was considerably reduced compared to less mobile protons of macromolecules constituting the dry soft tissue matter, characterized by a longitudinal relaxation T1 parameter that was shorter than for seawater protons. Seawater protons were also characterized by a longer transverse T2 relaxation parameter: subtraction of the intensity of a longer echo time, such as the 64 ms echo time, strongly weighted by the seawater protons from the intensity of the first echo time, provided an indicator that was correlated with the quantity of protons in the macromolecules of the dry soft tissue matter. However, Figure 2 shows that the dry weight of oysters with high gonad development could be slightly underestimated. This could be related to the effects of differences in the biochemical composition of soft tissue: gonad tissues are high in lipids (Matus de la Parra et al. 2003) which have higher proton density than glycogen and proteins. Clearly this method would be less precise than the gravimetric method based on lyophilization, but the new method has the advantage of being fast and easy to use for routine investigations and its non-destructive nature should allow precise individual follow-up studies of large oyster collections.

The NMR technique also appears to be able to provide an acceptable determination of ISC that can be used to calculate a condition index. ISC is clearly correlated with the intensity of the first echo of the non-weighted CPMG-15000 relaxation curve which indicates the quantity of all the protons from soft tissues and seawater in the NMR probe. The main difficulty is being sure that

the internal shell cavity volume is full of seawater and that shell valves remain closed during NMR scanning. An NMR sensor with a horizontal probe would be more appropriate to reduce the risk of water loss. Early methods sought to measure the internal shell cavity by volumetric methods but these are slow and difficult to perform accurately. A faster and easier gravimetric method was also developed: shell cavity capacity was well correlated with the difference between whole oyster weight and empty shell weight after drying for 24 h (Laurence and Scott, 1982). However, with this method, any water contained within the shells themselves (not between them) was included in the internal shell cavity weight (Abbe and Albright, 2003). The same limitation exists with the NMR method which quantifies all the protons contained in the NMR probe. Differences in shell morphology and fouling community structure may influence shell porosity and reduce the precision of the method.

Exploitation of all the data originating from both CPMG relaxation curves by more sophisticated chemometric methods provided encouraging results that also showed the good potential of NMR to determine sex and identify gonad development. However, given the small number of individuals and the difficulty in visually appreciating gonad development, further studies with larger sample groups are required to validate the method. LDA performed on individuals with dry tissues weighing over 0.7g provided a success rate of 100%. This clearly shows that the precision of the NMR method would be lower for small individuals. The diameter of the NMR probe used in this study did not allow scanning of larger oysters, particularly oysters in their second or third year of maturity. Further studies are required to design an NMR probe that is appropriate for bigger oysters and to improve sex discrimination and prediction of gonad development with larger groups. The method to determine soft tissue weight is rapid and probably sufficiently precise to investigate even small animals. The determination of internal shell cavity, sex and gonad development requires the acquisition of a complementary NMR signal based on a CPMG sequence with a long RD parameter. At the present time, this increases the acquisition time by 7 minutes. It would probably be advisable to reduce the overall

acquisition time by half and it should be possible to replace the non-T1-weighted CPMG-15000 sequence by a slightly T1 weighted sequence with RD between 5000 and 8000ms to achieve this. Another possibility would be to reduce the accumulation number (16 in the present study), particularly for the largest oysters which deliver the strongest signal originated from soft tissues.

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Captions for figures

Fig.1. Prediction of dry flesh weight by subtraction of the intensity of the echo at 64 ms from the beginning of the CPMG-400 relaxation curve from the intensity of the first echo. Effects of gonad development stages (0, 1, 2, and 3). 3N individuals were classified as stage 0.

Fig.2. Prediction of sex (3N triploid asexual oysters, 2N males and females) by discriminant functions originating from chemometric LDA method.

Table 1

Table 1. Parameters of **NMR** sequences

NMR CPMG sequence	RD	TE	Accumulation number	Acquisition time
CPMG-400	15000 ms	2 ms	16	6 min 51 s
CPMG-15000	400 ms	1 ms	32	47 s

Table 2. Prediction of quantitative phenotype characteristics, dry flesh weight (DW), fresh flesh weight (FW) and internal shell cavity (ISC) using simple or bilinear relationship with NMR data.

NMR data	Echo and Echo combination	DW		FW		ISC	
		R ²	RMSEP	R ²	RMSEP	R ²	RMSEP
CPMG-400	echo1	0.78		0.83		0.89	0.92g
	echo1-echo64	0.94	0.100g	0.91	0.54		
CPMG-15000	echo1	0.44		0.53		0.95	0.62g
	echo1-echo34	0.91	0.116	0.89	0.56		

RMSEP means Root Mean Square Error of Prediction

Table 3. Prediction of quantitative phenotype characteristics, dry flesh weight (DW), fresh flesh weight (FW) and internal shell cavity (ISC) using latent variables (LV) originating from chemometric PLS method

NMR data	Latent Variables	DW		FW		ISC	
		R ²	RMSEP	R ²	RMSEP	R ²	RMSEP
CPMG-400	3 LV	0.96	0.093g	0.94	0.52g	0.93	0,78g
	2 LV						
CPMG-15000	6 LV	0.95	0.116g	0.92	0.58g	0.98	0.67g
	5 LV						
CPMG-400-15000	5 LV	0.96	0.110g	0.94	0.53g	0.99	0.65g
	5 LV						

RMSEP means Root Mean Square Error of Prediction

Table 4. Prediction of sex (3N, 2N males and females) using discriminant functions originating from chemometric LDA method.

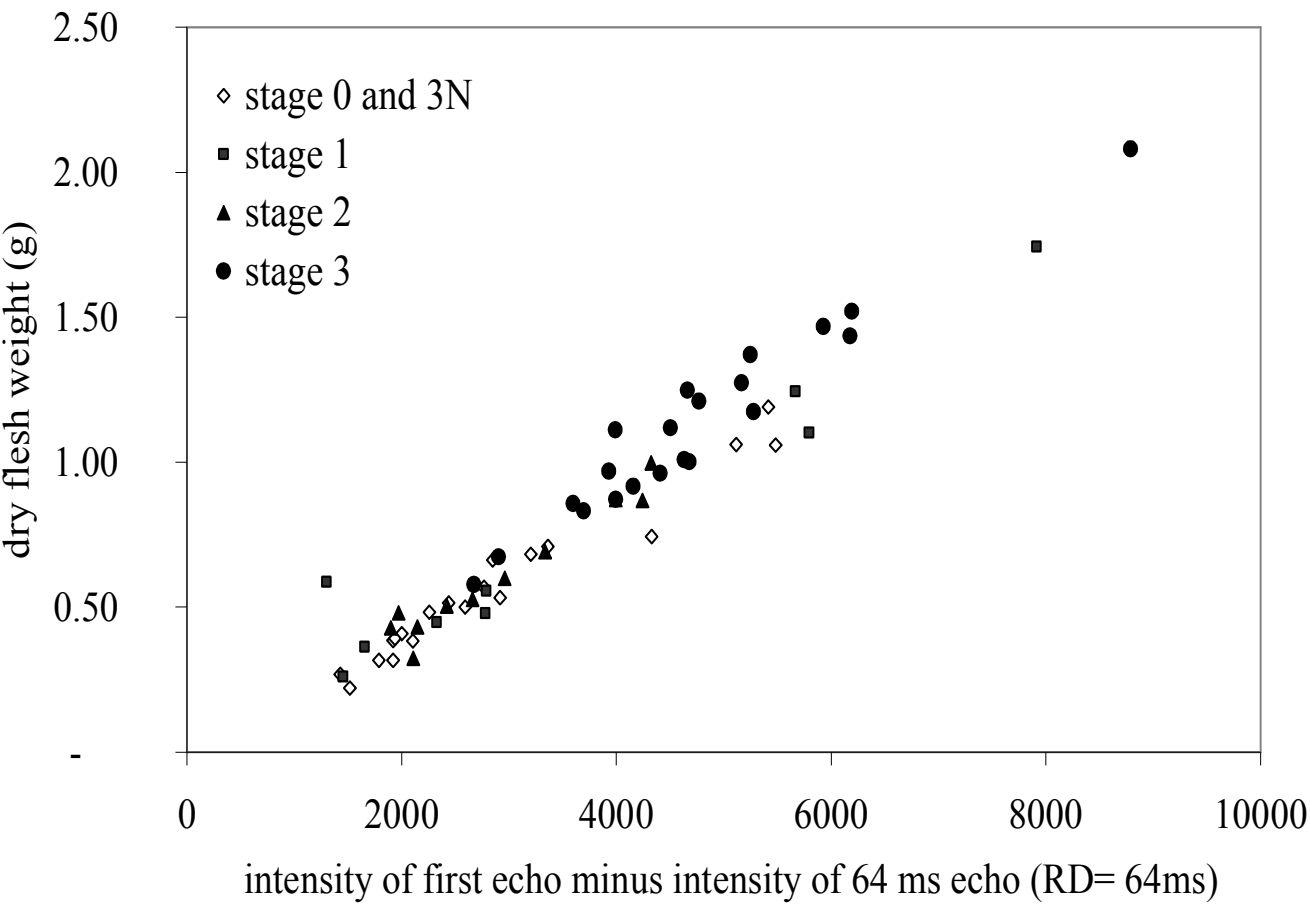
« Sex » observed	n	« Sex » predicted		
		3N	F	M
3N	25	25 (100%)	0	0
F	15	0	14 (93%)	1
M	16	1	2	13 (87%)

Table 5. Prediction of gonad development (GD) stages (1, 2, 3) of 2N males and females using discriminant functions originating from chemometric LDA method

GD observed	n	GD predicted		
		1	2	3
1	2	1 (50%)	1 (50%)	0
2	10	0	7 (70%)	3 (30%)
3	19	0	4 (20%)	18 (79%)

Figure 1

Fig.1.



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Reviewers' Comments	Corrections or authors' comments
Reviewer 1	
These authors may not mentioned in their publications related to NMR or MRI methods, if these non destructive techniques can have some impact, even very small, on different fecundity parameters (success of the spawning, quality of gametes, number of gametes and larvae) compared to animals in the same batches that have not been investigated in vivo MR imaging.	At the present time, we have not already realized such comparison to evaluate the impact of these non destructive methods on some fecundity parameters. However we have realized individual tracking of group of oysters until five temporal MRI scanings without mortality. We will take into account its valuable suggestion in the future.
Reviewer 2	
Line 35. Gigas, comma is underline.	Comma have been deleted in the text
Line 51.I will propose to add Magnetic resonance imaging "Magnetic Resonance (NMR) and Magnetic resonance imaging (MRI)" to avoid the confusion between the two techniques. Moreover, the authors have published before on MRI and not on NMR.	The proposal has been integrated in the text
Line 92: Do the authors can precise where is localised this NMR equipment?	Location has been added in the text
Lines 152-155. I was little confused with the first paragraph of the results. The authors must precise here, that these relationships concern only data obtained using either standard methods or NMR measurement?	These relationships concern only data obtained using standard methods: further details have been added in the text.
Line 211. Why the authors do not insert the animal in agar gel for ensuring that the shell valves remaining closed during NMR scanning.	In future experiments, we will take into account the interesting suggestion of the reviewer.
I especially recommend the authors to precise quantitative parameters concerning the following progenies obtained with the animals tested by NRM techniques. I will appreciate that these data could be included either in this manuscript if the authors have already some informations or in their future studies.	We have no information concerning the following progenies. We will include these data in our future experiments.
Fig. 2. I will suggest to encircle the three prediction of sex to be more in evidence	We have adopted this suggestion.
Table 2 and 3. Why some data are highlighted in bold type? RMSEP must be explain in the legend of the table.	Bold type has been deleted. RMSEP has been defined in the legend
Reviewer 2	
	No corrections asked