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Cell therapy medicinal product regulatory framework in Europe and its application for MSC-based therapy development

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Advanced therapy medicinal products (ATMPs), including cell therapy products, form a new class of medicines in the European Union. Since the ATMPs are at the forefront of scientific innovation in medicine, specific regulatory framework has been developed for these medicines and implemented from 2009. The Committee for Advanced Therapies (CAT) has been established at the European Medicines Agency (EMA) for centralized classification, certification and evaluation procedures, and other ATMP-related tasks. Guidance documents, initiatives, and interaction platforms are available to make the new framework more accessible for small- and medium-sized enterprises, academia, hospitals, and foundations. Good understanding of the centralized and national components of the regulatory system is required to plan product development. It is in the best interests of the cell therapy developers to utilize the resources provided starting with the pre-clinical stage. Whilst there have been no mesenchymal stem cell (MSC)-based medicine authorizations in the EU, three MSC products have received marketing approval in other regions since 2011. The information provided on the regulatory requirements, procedures, and initiatives is aimed at facilitating MSC-based medicinal product development and authorization in the EU.

Keywords: advanced therapy medicinal product, cell therapy medicinal product, mesenchymal stem/progenitor cell, Committee for Advanced Therapies, Hospital Exemption, national competent authority

INTRODUCTION

The scientific progress and advances in the biotechnology sector have led to the development of therapies which are based on the use of living cells, recombinant genetic material, and *in vitro* engineered tissue. A number of cell therapy and tissue engineered products have been introduced into the national markets of several Member States during the last decade. Due to the novelty, complexity, and technical specificity of such products, specially tailored and harmonized rules were necessary to ensure free movement of those products within the EU. Consequently, the Regulation (EC) N° 1394/2007 on advanced therapy medicinal products (ATMPs) was drafted and came into force on December 30, 2008. The Regulation laid down specific rules concerning centralized authorization, supervision, and pharmacovigilance of the ATMPs (Committee for Advanced Therapies and CAT Scientific Secretariat, 2010).

The term "advanced therapy medicinal product" covers the following medicinal products for human use: somatic cell therapy medicinal products (CTMPs), gene therapy medicinal products, and tissue engineered products. Combined ATMPs incorporate one or more medical devices as an integral part of the product. The scope of this article is primarily CTMP. For cells to be classified as medicinal products they have to fulfill at least one of the following conditions: the cells have been subject to substantial manipulation and/or these cells are not intended for use for the same essential function (the term "non-homologous use" is also used). By "substantial manipulation" it is understood that the biological characteristics, functions, or properties relevant for the therapeutic effect have been altered. Taking into account the methodological complexity of the cell therapy products, and in order to reduce the possible interpretations, it has been defined that certain manipulations with the cells and tissues are not to be considered as substantial. These include (tissue) cutting, grinding, shaping, centrifugation, soaking in antibiotic or antimicrobial solutions, sterilization, irradiation, cell separation, concentration or purification, filtering, freezing, cryopreservation, and vitrification (all listed in the Annex I of the Regulation (EC) N° 1394/2007). From the scientific or clinical perspective it can be argued that cell irradiation, for instance, can have a substantial effect on the biological characteristics and physiological functions that may be relevant also for the intended therapeutic application. Since interpretations by product developer and the regulator may differ, the exact legal definition for somatic CTMP is provided in **Table 1**.

The requirements for the cell therapy product marketing authorization dossier are prescribed in the Directive 2001/83/EC. It has to be verified whether the text of the directive includes the amendments introduced until at least 2011. A link to the consolidated version is provided in the references. Thus, cell therapy requirements were introduced in 2010 by the Directive 2009/120/EC amending the Directive 2001/83/EC. In brief, the particulars and documents of a cell therapy dossier are presented as five modules: Module 1 provides the European Community specific administrative data; Module 2 provides the quality, non-clinical, and

Table 1 | Glossary of key terms for advanced therapy developers in the EU.

Advisory procedures available for ATMP development process:

(a) *Classification* of product by the EMA: optional incentive for applicants, fast procedure, applied preferably during the early development stage;

(b) EMA *Innovation Task Force (ITF) briefing meeting*: early dialog with product developers with confidential and legally non-binding advice; the NCAs of some Member States may also provide similar "introductory" meetings with regulatory experts;

(c) *Certification* of quality and non-clinical data by the EMA: preferably applied before the clinical development stage, but the presence of clinical data does not preclude this procedure;

(d) *Scientific Advice* procedure: can address any quality, non-clinical, and clinical question at any time point of the product development (post-marketing advice is also available); provided by the EMA, but similar procedures can be offered in some countries by the national competent authority (NCA).

Advanced therapy medicinal product (ATMP)

Any of the following medicinal products for human use:

(a) gene therapy medicinal product,

(b) somatic cell therapy medicinal product,

(c) tissue engineered product.

Depending on the product characteristics, ATMP can be placed on the market in accordance with centralized marketing authorization procedure according to Regulation (EC) N° 1394/2007 or, if applicable to product, via Hospital Exemption clause in individual Member State.

Somatic cell therapy medicinal product (CTMP)

ATMP which has the following characteristics:

(a) contains or consists of somatic cells or tissues that have been subject to substantial manipulation so that the biological characteristics, physiological functions, or structural properties relevant for the intended clinical use have been altered; or of cells or tissues that are not intended for the same essential function(s) in the recipient and the donor;

(b) is presented as having properties for, or is used in or administered to human beings with a view to treating, preventing or diagnosing a disease through the action of its cells or tissues.

Centralized marketing authorization (MA)

The centralized MA procedure is required for certain categories of medicines in Europe, including the ATMPs. This procedure results in a single marketing authorization that is valid in all EU countries, as well as in Iceland, Liechtenstein, and Norway. The European pharmaceutical regulatory framework is applied, in particular the requirements of the Directive 2001/83/EC and the Regulation (EC) N° 726/2004, also the Regulation (EC) N° 1394/2007 with regard to the ATMPs. The EMA is responsible for the centralized procedure for medicines and the Committee for Advanced Therapies (CAT) evaluates ATMP submissions.

In order to address serious unmet medical needs of patients, it may be possible to obtain MA on the basis of less complete data than normally. Besides a standardized marketing authorization some medicinal product indications may present a case for a conditional approval. *Conditional marketing authorization* is subject to specific post-marketing obligations as set out in the Regulation (EC) N° 507/2006. However, such an authorization is not supposed to remain conditional indefinitely. Once the missing data are provided, it should be possible to replace it with a marketing authorization which is not conditional.

Hospital Exemption (HE)

Centralized MA is not required for such ATMPs which are prepared on a non-routine basis according to specific quality standards and are used within the same Member State in a hospital under the exclusive professional responsibility of a medical practitioner in order to comply with an individual medical prescription for a custom-made product for an individual patient.

Manufacturing of such ATMP products is authorized by the NCA of the Member State. The Member States have to ensure compliance with the adequate quality standards, as well as the traceability and pharmacovigilance requirements. The HE clause is implemented through the national legislative acts and there are differences among the HE regulations of individual Member States.

Transitional period

Advanced therapy medicinal products, excluding tissue engineered products, which were legally on the markets of the Member States in accordance with the or the EU legislation on December 30, 2008, had to comply with the Regulation (EC) N° 1394/2007 no later than by December 30, 2011. Tissue engineered products have to comply with the regulation no later than by December 30, 2012.

clinical summaries, Module 3 provides chemical, pharmaceutical, and biological information, Module 4 provides the non-clinical reports, and Module 5 provides the clinical study reports. The Regulation (EC) N° 1394/2007 also introduced amendments to the Directive 2001/83/EC, for instance Article 28 added provisions for the Hospital Exemption (HE, Article 3.7. of the consolidated Directive 2001/83/EC).

OVERVIEW OF THE EUROPEAN REGULATORY FRAMEWORK

Directives and regulations are the two types of the EU legislative acts that form the regulatory framework for all medicines, including cell-based products. This legal framework provides the basis for centralized and national competencies. The directives set the general requirements for the Member States which implement these requirements by adopting national legislative acts. Certain variability of these implementation measures exists amongst the Member States. The regulations have to be implemented directly and uniformly, without the national legislative acts. The Regulation (EC) N° 1394/2007 provides the legal basis for a centralized authorization procedure of the ATMPs – it involves a single scientific evaluation of the quality, safety, and efficacy of the product carried out to the highest possible standard by the European Medicines Agency (EMA). National (NCA) and centralized [EMA/Committee for Advanced Therapies (CAT)] competences at the different stages of cell therapy product development are summarized in **Table 2**.

Centralized procedures are provided by the EMA which is an interface for the cooperation and coordination of the activities of all 27 Member States with respect to the medicinal products. It is responsible for coordinating the existing scientific resources for the evaluation, supervision, and pharmacovigilance of the medicinal products. The EMA has seven committees including the CAT as well as a number of working parties which are expert groups with a specific scope and mandate. In addition to the evaluation of product marketing applications, the EMA mandates include scientific and procedure advice, the Innovation Task Force (ITF) meetings with product developers, coordination of the inspections of the Member States (GMP, GCP, GLP), and other. The mandates of the EMA do not cover the following ATMP development-related issues: pre-clinical development, clinical trial authorization, products legally on the market during the transitional period, HE authorization, functions of the ethics committee (but the EMA has the expertise to evaluate ethical issues), pricing and reimbursement of medicinal products. These issues are regulated at the Member State level by the national competent authority (NCA). Some states have one regulatory office whilst others have several NCAs that cover different regulatory tasks. The respective EU directives set the scene for the national regulatory frameworks. The Directive 2004/23/EC (with the implementing Directives 2006/17/EC and 2006/86/EC) defines the quality and safety standards for the donation, procurement, testing, processing, preservation, storage, and distribution of human tissues and cells. In the case of blood cells or blood components for the ATMP manufacture, the requirements of the Directive 2002/98/EC apply. For ATMPs that contain human cells or tissues, Directive 2004/23/EC and 2002/98/EC derived national provisions will apply as far as donation, procurement and testing are concerned. The requirements of these directives do not apply to research projects, their scope is only the tissues and cells intended for human use. Clinical trials with ATMPs should be conducted in accordance with the overarching principles and the requirements laid down in the Directive 2001/20/EC on approximation of the national laws, regulations and the administrative provisions for the implementation of good clinical practice in the conduct of clinical trials on medicinal products for human use. Compared with the regulatory system in the US, the EMA is not "FDA of Europe" since the FDA has a direct mandate to regulate the above mentioned issues and perform other tasks, including research. The current developments indicate that the EMA is likely to acquire more mandates in the future which will reduce the historical fragmentation of the European regulatory framework for medicinal product development.

The CAT provides a centralized cell therapy product evaluation procedure. The CAT formulates a draft opinion on the quality, safety, and efficacy of a product for the final approval by the Committee for the Medicinal Products for Human Use (CHMP). The EU marketing authorization which is based on a centralized evaluation procedure takes 210 days excluding clock-stops, it is defined in the Regulation (EC) N° 726/2004. The evaluation is done by two independent (Reporter and Co-Reporter) assessor

Table 2 | Centralized and national components of the regulatory framework for ATMP development in the EU.

Type of activity	Legislation	NCA	EMA/CAT
Cell and tissue donation, procurement, processing	National	Inspection, authorization	n/a
Pre-clinical development	National	GLP inspection, consultation	Certification procedure (optional)
Clinical development	National	GCP, GMP inspections, authorization	n/a
ATMP classification	EU	Consultation	Procedure/opinion
ATMP certification	EU	n/a	Procedure/opinion/certificate
ATMP evaluation	EU	n/a	Procedure/opinion
Transition period	EU	Consultation	ATMP evaluation
Hospital exemption	National	Consultation, GMP inspection, production license	n/a

GLP, good laboratory practice; GCP, good clinical practice; GMP, good manufacturing practice.

teams with the CAT and the CHMP representatives. The fee for marketing authorization is reduced by 50% if the applicant is a hospital or a small- or medium-sized enterprise and can prove that there is particular public health interest in the ATMP concerned. This is prescribed in the Article 19 of the Regulation (EC) N° 2007/1394. However, even if the marketing authorization for a cell therapy product is granted, the Regulation does not interfere with the decisions of the Member State on whether to allow the use of any specific type of human cells such as embryonic stem cells or xenogeneic cells. It does not affect the application of the national legislation prohibiting or restricting the sale, supply or use of medicinal products containing, consisting of or derived from particular cells. Several cell therapy products were legally on the Member State markets before December 30, 2008. Such products have been granted a transition period defined in the Article 29 of the Regulation (EC) N° 1394/2007 during which products have to comply with the Regulation. The transition period for CTMPs has already expired at the end of 2011, and for tissue engineered products the transition period expires in 2012.

The evaluation-related tasks of the CAT include the classification of the advanced therapy products and certification of their pre-clinical data quality. Product developers have access to the classification procedure in order to determine whether a given product based on cells, genes or tissues meets the scientific criteria which define it as an ATMP. It is an incentive but not a legal requirement for the applicants and an opinion is delivered within 60 days after the receipt of the request. More than 50 classifications have been completed by 2012 and non-confidential summaries are available on the EMA website. ATMP classification procedure does not determine whether product dossier will be evaluated by the centralized procedure in or it can be submitted for the HE in Member State. However, the CAT classification procedure opinion is not legally binding for the NCAs in case product is submitted for the HE. Finally, certification of the quality and non-clinical data is a new and unique procedure available only for the medicinal products of advanced therapy classification and it is based on the Regulation (EC) N° 668/2009. Micro businesses and SMEs developing an ATMP can submit all the relevant quality and where available non-clinical data to the EMA for scientific evaluation by the CAT. It is a 90-day procedure and in the case of a favorable CAT opinion the EMA will issue a corresponding certificate. The certification is not legally binding but it will facilitate the development and improve the clinical trial and marketing authorization applications based on the same data. Only one cell therapy certification has been completed since the launching of this procedure. This is possibly due to the optional nature of the procedure and the interpretation that the resulting opinion is not legally binding for the EMA. It is also possible that SMEs developing cell therapy products may not be yet fully aware of this procedure and the related potential benefits. It has to be emphasized that a positive outcome of the certification procedure indicates that the regulatory agency has evaluated and recognized the quality of non-clinical data. Certification of the data quality also minimizes the possibility of major objections at the marketing application evaluation stage and may serve as an incentive for investments in the development of cell therapy.

Upon request of the Executive Director of the EMA or the European Commission the CAT provides advice and scientific support to drafting documents related to the fulfillment of the objectives of the ATMP regulation. On request of the European Commission the committee provides scientific expertise and advice for initiatives related to the development of innovative medicines and therapies which require the expertise in advanced therapy-related scientific areas. The activities proposed in the CAT Work programme 2010–2015 may be of interest for the ATMP stakeholders. The document is available at the EMA website and link is provided in the references. Considering the potential of the ATMPs and due to the lack of product progress to the market, the CAT has adopted proactive approach in providing the guidance tools and ensuring a dialog with the relevant parties. A number of program activities are targeted at the needs of the ATMP developers and the stakeholders are welcome to communicate their opinion via the EMA website. The CAT Work programme for the period of 2010–2015 is aimed at providing positive long term impact on the advanced therapy sector in Europe.

CONSIDERATIONS FOR DEVELOPMENT OF MSC-BASED MEDICINES

The mesenchymal stem cells (MSCs) have produced beneficial effects in a wide range of pre-clinical development disease models, even though there are as yet no adequate explanations for many of the effects observed (Prockop and Oh, 2012). During the last decade the MSC-based therapy clinical trials have been conducted for at least a dozen of different medical conditions (Wang et al., 2012). The results of clinical studies have led to the conclusion that MSC applications have been safe and feasible. However, the efficacy often could not be convincingly demonstrated as the therapies advanced along with the clinical development. This is also illustrated by the absence of MSC-based products in the European market. Only a few MSC-based cell therapy products have been approved in other markets worldwide. South Korea is leading with two MSC products registered and the first authorization granted in 2011. It might be linked to the procedure of a conditional marketing approval in the regulatory framework of South Korea that allows commercial sale in certain instances whilst pivotal trials are underway. There is also a procedure of conditional marketing authorization in the EU prescribed by the Regulation (EC) N° 507/2006 but certain differences exist in the regulatory systems. With the approval from the Korean FDA in January 2012, Cartistem has become the world's first allogenic, off-the-shelf MSC-based product. The product contains the umbilical cord blood (UCB)-derived MSCs and it is indicated for the treatment of traumatic and degenerative osteoarthritis. In 2011 the Korean company FCB PharmiCell received Korean FDA approval for commercial sale of HeartiCellgram indicated for post-acute myocardial infarction treatment. It is autologous bone marrow-derived MSC therapy product. The company provides 50–90 million cells (depending on the weight of the patient) which are administered by infusion into the coronary arteries. The regulatory approval for HeartiCellgram was granted after 6 years of clinical trials. The company has announced that the patients displayed a 6% improvement in the left ventricular ejection fraction 6 months after one dose of HeartiCellgram. However, the company has not published the results in a peer-reviewed journal (Wohn, 2012). It seems that a similar regulatory decision has been adopted for Osiris Therapeutics Inc. product Prochymal which consists of allogenic MSCs. The company was granted an authorization for the treatment of acute graft-vs-host disease (GvHD) in children under Health Canada's Notice of Compliance with conditions (NOC/c) in May 2012. This is an authorization to market on condition that the manufacturer undertakes additional studies to verify the clinical benefit. Such a regulatory pathway provides access to treatments for unmet medical conditions and has demonstrated the benefits outweigh the risks in the clinical trials. Overall this may represent a regulatory trend to consider the evaluation procedures that could address medical needs more efficiently. Adaptive licensing, e.g., conditional approvals, would be based on stepwise learning in circumstances of acknowledged uncertainty, with iterative phases of data gathering and regulatory re-evaluation (Eichler et al., 2012). Adaptive licensing requires a different approach from the standardized dichotomous unapproved/approved product paradigm.

Better understanding of the regulatory framework should improve the development strategy and create opportunities for MSC-based therapy product authorization also in Europe. From the regulatory perspective all MSC-based products in the EU will be classified as ATMPs unless the developer claims that the MSCs have been obtained without *in vitro* culture step. According to the CAT opinion, the cell culture process corresponds to a "substantial manipulation" and the derived cells qualify as an active substance of a medicinal product. The MSCs containing medicinal product can be classified as cell therapy or tissue engineered product depending on the intended use and the claimed mode of action. The addition of recombinant proteins, chemicals and biologically active molecules *in vitro* to the MSCs will not change the regulatory status of the product. Introduction of gene expression vector into MSCs does not change the ATMP status but will result in reclassification from the cell to gene therapy product, since the product which may fall within the definition of the CTMP and the gene therapy medicinal product should be considered as a latter (as defined in Part IV of Annex I to Directive 2001/83/EC). The studies that report MSC clinical application often present the development strategy decisions which could be re-evaluated in case the overall aim was to develop a cell therapy product. It is often reported that unmodified primary MSC cultures have been used. Correspondingly, pre-clinical screening has been absent since there was only one active substance candidate. The rationale of the use of a particular MSC culture should be evaluated if mesenchymal cell clinical application does not require the same essential function as in the tissue of origin. Instead, the MSC trials often aim to facilitate the tissue regeneration or to achieve the immunomodulatory effect. Pre-clinical screening and selection is an integral part of conventional medicinal product development and there is no reason to assume that it should not be introduced also for the cell therapy developments. With the technology available there are several strategies that might be considered in order to introduce the MSC therapy candidate selection step. For instance, modification of a primary MSC population with small chemical compounds and subsequent screening for expression or secretome profiles could be considered to improve the study design and the efficacy (Ranganath et al., 2012). Genetic modification to express factor(s) that are expected to mediate or enhance the therapeutic effect and characterization of transfected clones also represents rational development (Olson et al., 2012). The comparison of subpopulations or primary cultures of different origin will increase the number of candidates for the screening and has been applied for the cell-based medicinal product development (Li et al., 2012). These examples illustrate the feasibility of the pre-clinical screening step also for the cell-based therapies. Whilst the screening will increase the costs and may not be attractive or necessary for the academic research activities, it may present a cost efficient improvement for the discovery of new MSC therapy candidates. It may provide benefits also from novel intellectual property acquisition perspective.

The MSC product developers are advised to start with the EMA scientific guidelines that provide a detailed description of the quality, safety, efficacy, and pharmacovigilance issues for CTMPs. These guidance documents include the "Guideline on human cell-based medicinal products," "Guideline on the safety and efficacy follow-up – risk management of advanced therapy medicinal products," "Guideline on strategies to identify and mitigate risks for first-in-human clinical trials with investigational medicinal products," "Guideline on the quality, preclinical and clinical aspects of medicinal products containing genetically modified cells," and "Reflection paper on stem cell-based medicinal products." "Guideline on the risk-based approach according to Annex I, part IV of Directive 2001/83/EC applied to Advanced Therapy Medicinal Products" is an important ATMP guidance document which has been published for public discussion and will be finalized by the end of 2012 (the e-links for all guidance documents are provided in the references). The above-mentioned guidance documents should be examined in detail but it is neither the scope of these documents nor is it technically feasible to describe the whole variety of cell therapy products. Instead, the risk analysis may cover the entire development process and an adequate risk-based approach strategy has to be applied. According to the Directive 2001/83/EC, due to the specific nature of the ATMPs, the application of a risk-based approach is encouraged to determine the extent of quality, the non-clinical and clinical data to be included in the marketing authorization application. The risk analysis methodology followed, the nature of the identified risks and the implications of the risk-based approach for the development and evaluation program has to be discussed and the risk analysis has to be described in the product application. For instance, if conventional pharmacological and toxicological tests may not be considered as appropriate for a cell therapy product, the relevant biological parameters such as the cell viability, biodistribution, ectopic growth, and the expression patterns can be investigated. It is acknowledged that relevant animal models for cell therapy product and indication might not exist. Immunocompromised animals may have a limited value, the structural and functional dissimilarities between the animal and human target organs/tissues may not produce relevant data. It would be reasonable to assume that the marketing authorization and certification application evaluation will be carried out in accordance with the risk-based approach until a certain number of products reach the market.

The whole range of assistance procedures and initiatives available from the EMA and the NCAs should be considered (summary in **Table 1**). The product classification procedure is highly recommended at an early stage of development. This will confirm the legal framework and the relevant guidance documents can be applied for the development. Certification of non-clinical and quality data should be considered since a positive outcome would facilitate and streamline the product development for authorization. Since there are no MSC products authorized in the EU, certification by the regulatory agency may encourage funding agencies and the potential investors. Application for EMA ITF briefing meeting definitely should be considered. The ITF provides a multidisciplinary group that contains scientific, regulatory, and legal competences. It is a forum for an early dialog with the applicants. The ITF briefing meetings are meant to complement other regulatory procedures, such as the classification, certification, and scientific advice (SA). The ITF meetings are free of charge; the application forms and information about the procedure are available at the EMA website. The SA procedure provides considerably more detailed information to the applicant and can be used at any stage of the MSC product development. The procedure helps the applicant to make sure that appropriate tests and studies are performed. Consequently no major objections are likely to be raised during the evaluation of the marketing authorization application. Such major objections could significantly delay the marketing of a product and may result in a refusal of the authorization. Adherence to the SA recommendations can substantially increase the probability of a positive marketing outcome (Regnstrom et al., 2010). Within the current initiative the EMA provides SA to the SMEs for a fee reduced by 90%, and with a 65% reduction for other applicants that develop the ATMPs. Several NCAs also have the capacity and the expertise to provide similar advice but this has to be confirmed with the particular agency. The protocol assistance procedure should be considered for orphan or rare disease products, i.e., a condition affecting no more than 5 in 10,000 people in the EU. This procedure is available at the EMA and presents a special form of SA. As a result the "orphan designation" may be applied for medicinal products that meet the criteria and the incentives for "orphan designation" include the fee reduction and 10 years of market exclusivity once authorized. Products developed for ultra rare diseases may qualify for a marketing authorization under exceptional circumstances which requires a less complete data set. The following guidance document has been published for further information: "Guideline on procedures for the granting of a marketing authorization under exceptional circumstances, pursuant to Article 14 (8) of Regulation (EC) N° 726/2004."

Alternatively, an MSC therapy product may be considered for national authorization procedure under the HE clause in a particular Member State. In order to qualify for HE authorization, a product has to be prepared on a non-routine basis according to specific quality standards, and has to be used within the same Member State in a hospital under the exclusive professional responsibility of a medical practitioner (legal definition provided in **Table 1**). The HE regulatory instrument could be in a way perceived as adaptive licensing at the national level for advanced therapy medicinal products. It has to be acknowledged that inherent and unavoidable autologous cell material differences can make certain cell therapy applications more similar to the development of medical technology than to classical medicinal product development process with standard clinical trials (Webster et al., 2011). This does not mean that autologous cell products by definition would qualify for HE approval since regulatory decision is not based on the origin of cell material. However, autologous products combined with complex medical procedures are more likely to qualify for the HE due to the inherent product characteristics. In either case the HE is still a very new regulatory procedure which, if considered, should be discussed in advance with the experts of an NCA. The implementation of the HE clause has been accomplished in the majority of EU states by 2012, but the terms and conditions of the authorization vary and each Member State decides on the implementation tools. For instance, a recent publication illustrates the differences between France and the UK regarding the HE authorizations and also provides information on several other ATMP development-related issues that are regulated differently at the national level (Mahalatchimy et al., 2012). This reference should be examined in case the application for the HE authorization is planned in the UK or France. For instance, the UK agency provides options for either the HE authorization or the "Specials" exemption status according to Article 5.1. of Directive 2001/83/EC. The website of the UK NCA – Medicines and Healthcare products Regulatory Agency (MHRA) – provides user friendly information on the ATMP-related questions and relevant flowcharts in the section "How we regulate advanced therapy medicinal products." Information on the HE clause in Germany is available on the website of Paul-Ehrlich-Institute (PEI) which is the German NCA. The PEI Innovation Office website includes summaries in English and flow-chart on how the ATMPs are regulated in Germany (links are provided in references). German ATMP framework is reviewed and analyzed in detail in a recent publication (Buchholz et al., 2012).

CONCLUSION

Regulatory centralization has been introduced for cell therapy product marketing in the EU since 2009 but the remaining national procedures can be quite heterogeneous. Still, general provisions in the national legal acts are based on the EU directives and therefore will be common for all Member States. The EMA and NCA guidance documents, initiatives, and interaction platforms are available to make the regulatory framework more understandable and accessible for investigators both in the public and private sectors. Good understanding of centralized and national components of the framework will form an essential part of a product development plan and initial shortfalls will be difficult to compensate at the marketing authorization application stage. It is in the best interests of the investigators and investors to communicate with the NCAs and the EMA already during early phase of MSC product formulation. Resulting strategy improvements may facilitate MSC-based medicine development and authorization in the European Union.

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APPENDIX

LEGISLATIVE ACTS

Regulation (EC) N° 726/2004 of the European Parliament and of the Council of 31 March 2004 laying down Community procedures for the authorisation and supervision of medicinal products for human and veterinary use and establishing a European Medicines Agency. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2004:136:0001:0033:en:PDF

Regulation (EC) N° 507/2006 on the conditional marketing authorisation for medicinal products for human use falling within the scope of Regulation (EC) No 726/2004 of the European Parliament and of the Council. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:092:0006:0009:EN:PDF

Regulation (EC) N° 1394/2007 of the European Parliament and of the Council of 13 November 2007 on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) N° 726/2004. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2007:324:0121:0137:en:PDF

Commission Regulation (EC) N° 668/2009 of 24 July 2009 implementing Regulation (EC) No 1394/2007 of the European Parliament and of the Council with regard to the evaluation and certification of quality and non-clinical data relating to advanced therapy medicinal products developed by micro, small and medium-sized enterprises. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:194:0007:0010:EN:PDF

Directive 2001/20/EC of the European Parliament and of the Council of 4 April 2001 on the approximation of the laws, regulations and administrative provisions of the Member States relating to the implementation of good clinical practice in the conduct of clinical trials on medicinal products for human use. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2001:121:0034:0044:EN:PDF

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GUIDANCE DOCUMENTS

Guideline on procedures for the granting of a marketing authorisation under exceptional circumstances, pursuant to Article 14 (8) of Regulation (EC) N° 726/2004 (EMEA/357981/2005). Available at: http://www.ema.europa.eu/docs/en_GB/document_library/Regulatory_and_procedural_guideline/2009/10/WC500004883.pdf

Guideline on human cell-based medicinal products (EMEA/CHMP/410869/2006). Available at: http://www.emea.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003894.pdf

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Predator transitory spillover induces trophic cascades in ecological sinks

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Understanding the effects of cross-system fluxes is fundamental in ecosystem ecology and biological conservation. Source-sink dynamics and spillover processes may link adjacent ecosystems by movement of organisms across system boundaries. However, effects of temporal variability in these cross-system fluxes on a whole marine ecosystem structure have not yet been presented. Here we show, using 35 y of multitrophic data series from the Baltic Sea, that transitory spillover of the top-predator cod from its main distribution area produces cascading effects in the whole food web of an adjacent and semi-isolated ecosystem. At varying population size, cod expand/contract their distribution range and invade/retreat from the neighboring Gulf of Riga, thereby affecting the local prey population of herring and, indirectly, zooplankton and phytoplankton via top-down control. The Gulf of Riga can be considered for cod a "true sink" habitat, where in the absence of immigration from the source areas of the central Baltic Sea the cod population goes extinct due to the absence of suitable spawning grounds. Our results add a metaecosystem perspective to the ongoing intense scientific debate on the key role of top predators in structuring natural systems. The integration of regional and local processes is central to predict species and ecosystem responses to future climate changes and ongoing anthropogenic disturbances.

ecosystem regulation | predator distribution | landscape ecology | exploited resources | cross-system management

Ecological connectivity and spatiotemporal linkages among communities and ecosystems, as well as the interactions between regional and local processes, are fundamental aspects in ecology (1, 2) for the understanding of ecosystem functioning on a broader landscape (or metaecosystem) scale (3, 4). In particular, cross-habitat fluxes of organisms (mediated by passive transport or active migration) occur between natural habitats and across natural/anthropogenic systems, at various geographical scales (5–9). In heterogeneous landscapes, a particular case of cross-habitat fluxes is represented by source-sink dynamics in which a population from a productive source habitat maintains by migration populations in sink habitats where local reproduction is insufficient to compensate for mortality (10, 11). These processes are sometimes mediated by spillover, where an increase in population abundance in the source may result in a fast colonization of sink habitats (12, 13). The directional flow of organisms across systems boundaries has the potential to influence local food-web dynamics by coupling the trophic dynamics (e.g., predators and prey) of different habitats and ecosystems, as shown by theoretical and empirical studies (4, 14). In marine ecosystems, field examples of spillover effects have been presented mainly in terms of the effects that fishery no-take areas have on the external exploited fraction of the target populations (15, 16). Evidence of spillover impacts on the whole structure of pelagic marine ecosystems has until now not been documented in a natural source-sink dynamic context; with this study, we provide such an example linked to the management of marine resources.

Herein we applied a sequential modeling setup (17) using generalized additive models (GAMs) on a dataset of multiple trophic levels (from predatory fish to phytoplankton) and hydrological factors collected in the Baltic Sea during the past 35 y (Fig. 1 and Fig. S1). We show that the structure of a semi-isolated pelagic ecosystem [the Gulf of Riga (GoR); Fig. 1 and *SI Materials and Methods*] during summer is shaped by a series of top-down effects (i.e., a trophic cascade) (18), propagating from the neighboring Baltic Main Basin (MB). In the GoR the trophic cascade is modulated by the occasional occurrence of the main predatory fish of the Baltic Sea, the cod (*Gadus morhua*), which under specific conditions spill over from the MB.

Results and Discussion

The Baltic cod population rapidly increased in the late 1970s to record levels, due to a combination of particularly favorable hydrological conditions for reproduction, and relatively low levels of exploitation (19, 20). At the same time, the cod expanded its distribution into the northern MB, and spilled over into adjacent areas such as the GoR (Fig. 1*A*). The appearance and rise of cod in the GoR is demonstrated by scientific survey data and documented by the start of a cod fishery as shown by a progressive increase of commercial catches (Fig. 1*B* and Fig. S2). Cod is not able to successfully reproduce in the GoR due to the low salinities; its occurrence in the GoR is hence mediated by active migration of juvenile and adult individuals, as well as by passive larval dispersal, from the MB (21). The MB acts therefore as a source of cod that, during periods of high abundances, expands its distribution area and colonizes the sink habitat of the GoR.

From the mid 1980s, the Baltic cod population rapidly decreased and eventually collapsed (Fig. 1*A*), owing to overfishing and adverse hydrological conditions for its reproduction (19, 20). Concomitant with the decline in population size, the cod distribution contracted back to the southern Baltic Sea, where the hydrological conditions were more suitable for spawning and reproduction (i.e., high salinity and oxygen). As a result, the cod quickly disappeared from the GoR, and the commercial catches in this area dropped rapidly to zero (Fig. 1*B* and Fig. S2). The cod biomass index in the GoR was almost fully explained in our

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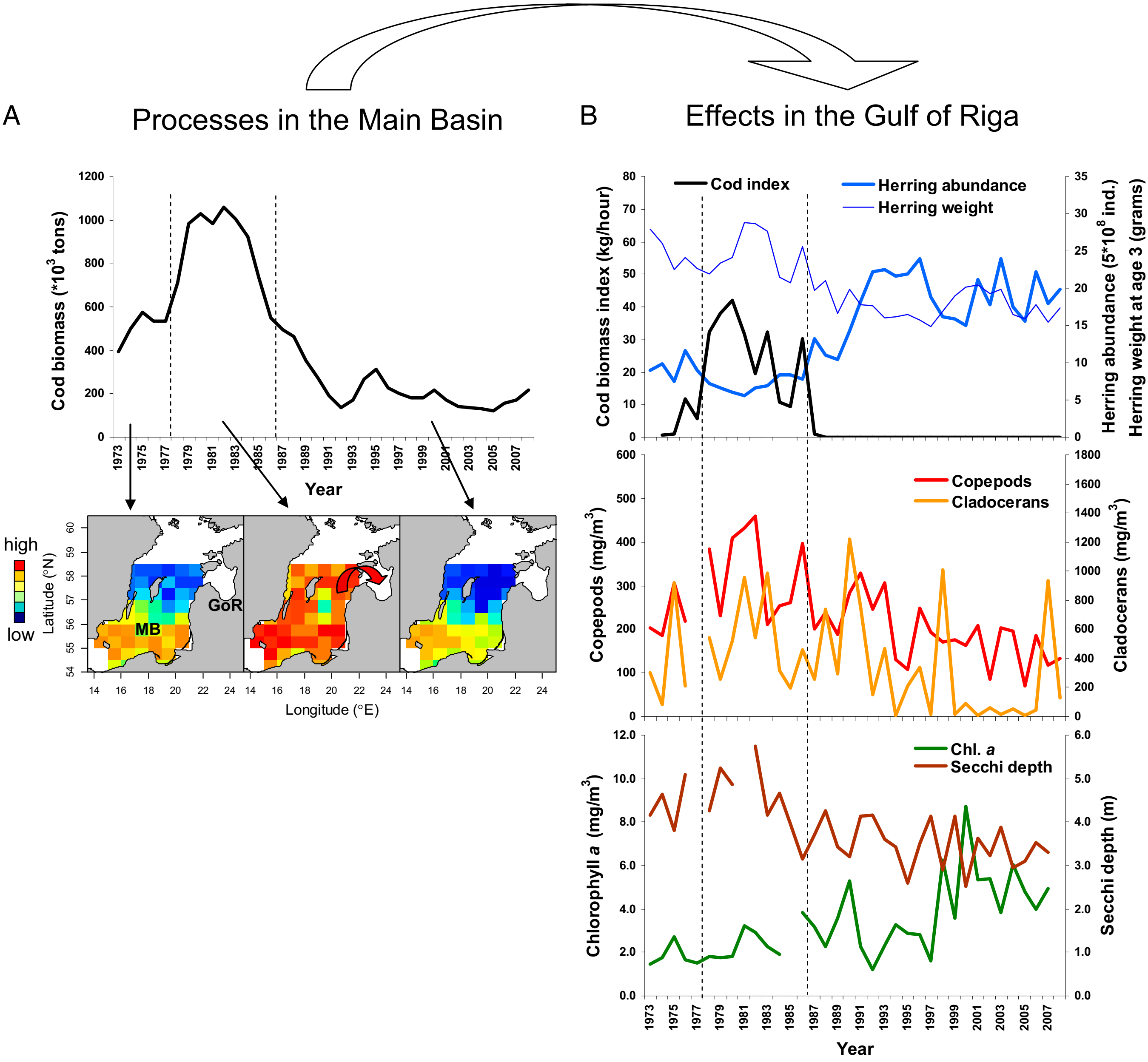

Fig. 1. Structural changes in the MB and GoR ecosystems during the past 35 y. (*A*) Changes in cod biomass and spatial distribution in the MB (source habitat for cod). (*B*) Changes in the food web of the GoR (sink habitat for cod), as indicated by time series of cod biomass index, herring abundance, zooplankton, and phytoplankton. The vertical dashed lines indicate the period of maximum cod population size and range of distribution in the MB that triggered the spillover into the GoR. The scale bar next to the distribution maps is in relative values.

model by cod biomass in the MB (deviance explained = 84.3%; Table 1, Fig. 2, and Fig. S3), providing quantitative evidence of the link between the two ecosystems at the top of the food web. Fishing mortality on the GoR cod has certainly accelerated its local decline when the immigration from the source area of the MB ceased.

The effects of the MB cod spillover and contraction propagated down the GoR food web, as suggested by the sequential modeling of each trophic level (Table 1, Fig. 2, and Fig. S3). The invasion and successive disappearance of the top-predator cod in the GoR was paralleled by a twofold decrease and then dramatic increase in the population of its main pelagic prey (i.e., herring; Fig. 1*B*). The variations in herring abundance were explained in our models mainly by changes in the cod biomass index, although fishing and spring temperature also have a significant effect (Table 1, Fig. 2, and Fig. S3)—the latter likely through enhanced recruitment (23). The relatively low herring population at the beginning of the 1970s was likely due to high fishing pressure (Fig. S2). The enduring high herring population from the early 1990s, which resulted despite an increase in fishing pressure, can instead be attributable to predation release from cod intertwined with an increase in spring water temperature of nearly 2 °C (Fig. S1).

Herring is the major zooplanktivore in the GoR ecosystem (*SI Materials and Methods*), and its population size was inversely correlated to the summer biomass of both copepods and cladocerans (Table 1 and Figs. 1*B* and 2). In addition to quantitative evidence of top-down regulation on zooplankton, temperature

Table 1. Results of the GAMs for each trophic level of the GoR

GAMs	Predictors	GCV	r^2 adjusted	Deviance explained, %	n	df	F	P	Difference deviance explained, %
Cod biomass, model A	Cod biomass MB					2.39	60.17	<0.0001	—
	Salinity, summer–autumn*								
	Temperature, summer–autumn*								
	Final model	0.398	0.83	84.30	35				
Herring abundance, model B	Cod biomass					1.00	93.65	<0.0001	20.37
	Fishing pressure					1.00	8.61	0.007	2.48
	Zooplankton, spring[†]								
	Temperature, spring[†]					1.00	5.14	0.031	1.40
	Final model	0.021	0.92	92.80	32				
Herring size, model B1	Herring abundance					1.65	27.70	<0.0001	—
	Temperature, spring–summer[‡]								
	Salinity, spring–summer[‡]								
	Final model	0.015	0.64	65.20	36				
Copepod biomass, model Ca	Herring abundance					2.08	8.34	<0.001	—
	Chl. *a* biomass, summer								
	Temperature, summer								
	Salinity, summer								
	Final model	0.129	0.38	41.80	35				
Cladoceran biomass, model Cb	Herring abundance					1.00	6.73	0.015	22.39
	Chl *a* biomass, summer								
	Temperature, summer					1.85	4.12	0.023	27.69
	Salinity, summer					2.83	5.37	0.005	45.47
	Final model	1.458	0.50	58.50	35				
Chl *a* biomass, model D	Copepod biomass, summer					1.00	7.33	0.011	30.14
	Cladoceran biomass, summer								
	River runoff[§]					1.00	5.75	0.023	27.75
	Temperature, summer					1.00	6.02	0.020	28.95
	Final model	0.098	0.36	41.80	33				

Difference deviance explained indicates the deviance contribution of the predictors in each model, which was estimated based on the percent difference in explained deviance of the final models after deletion of one predictor at a time while keeping all the rest (i.e. with replacement; ref. 22). GCV, Generalized Cross Validation. Fig. S3 shows the partial effects of the predictors in each model.

*GoR hydrological conditions after cod spawning in the MB.

[†] Copepod *Eurytemora affinis*, main prey for herring larvae, and temperature are taken during the herring spawning period.

[‡] Hydrological conditions during the main herring growth seasons.

[§] River runoff and nutrients concentration (dissolved inorganic nitrogen and dissolved inorganic phosphorus, both separated and summated) were separately tested as bottom-up forces, the second being not significant.

and salinity were also significant predictors for the cladocerans in our models (Table 1 and Fig. S3), confirming the importance of hydrological variability on zooplankton dynamics (24). Mean body size (weight at age 3) of herring was also negatively related with herring abundance, evidencing a strong density-dependent response to the changes in herring population size (Table 1, Fig. 2, and Fig. S3). Increased feeding competition within the enlarged herring population has likely been the mechanism mediating the depletion of zooplankton resources and the decrease in herring body size during the past three decades (Fig. 1*B*).

Top-down control was even detectable at the phytoplankton level [indicated by chlorophyll *a* (Chl *a*)], although its strength was minor compared with the higher trophic levels. This attenuation of top-down control at lower trophic levels conforms to what was found in other field and experimental studies (25). Though a significant negative effect of copepods on phytoplankton was unveiled in our model (26), temperature and river Daugava runoff were also important drivers (Table 1, Fig. 2, and Fig. S3), the latter likely subsidizing the semienclosed GoR ecosystem with nutrients (27). River runoff in GoR is eventually driven by large-scale climatic processes acting on precipitation and ice dynamics (28). The impact of top-down forcing on the summer phytoplankton biomass was also indirectly evidenced by the Secchi depth, an indicator of water transparency, which covaried positively with the copepods (Fig. 1*B*, Table S1, and Fig. S3).

The propagation of predator spillover and contraction effects through the GoR ecosystem was supported by the detection of indirect top-down regulation on trophic levels two or three steps below the predator. Specifically, cod biomass in the MB was a major predictor of herring abundance (negative effect), zooplankton biomass, herring body size, Secchi depth (positive effects), and phytoplankton biomass (negative effect) in the GoR (Table S2 and Figs. S4 and S5). These relationships unveil the occurrence of indirect mutualisms between trophic levels and reinforces the evidence of trophic cascades propagating across systems mediated by changes of cod distribution in the Baltic Sea landscape.

The dynamic between the MB and the GoR may represent an empirical example of a temporally heterogeneous source-sink dynamic (29), whose existence and persistence is regulated by the size and geographical distribution of the top-predator population in the source area. Cod fishery and habitat conditions, in terms of salinity and oxygen levels in the source habitat, are likely the major factors affecting the connectivity between the two systems, by limiting cod population size and distribution range (19). The GoR can be considered for cod as a true sink (29) or absolute sink (30), where in the absence of immigration from the source areas of the MB the cod population is not able to persist due to the lack of suitable spawning habitats. The linkage between the two systems is mainly mediated by active dispersal of juveniles and adult fish into the GoR when cod population size in the MB

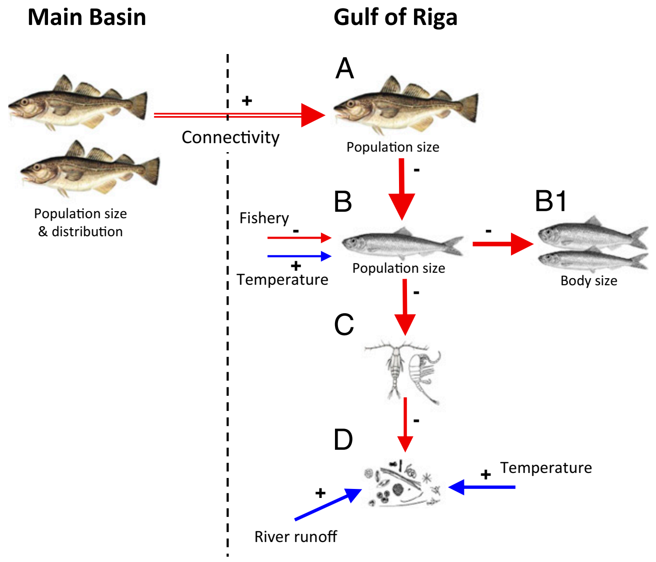

Fig. 2. Schematic representation of the effects of MB cod through the food web of the GoR. The thickness of the arrows indicates the relative deviance contribution of the predictors in each model (i.e., on each trophic level A–D and on herring body size B1). Red, top-down effects; blue, bottom-up and hydrological effects. The direction of the interactions is also indicated. Table 1 shows the statistics of the models, and Fig. S3 the partial effects of the predictors in each model. Cladoceran model is not shown because of the lack of relation with phytoplankton.

is high. In this case the GoR acts as nursery area for juveniles (31) and feeding area for both juveniles and adults of cod (21). During periods of high cod population size, its main pelagic food resource in the MB (i.e., the fish sprat) is depressed (17), and thus cod may search for alternative foraging habitats, as the GoR. This response would conform to the density-dependent ideal free-distribution theory (32), which suggests that as population size increases, individuals spread into less-favorable habitats. Also under these circumstances, however, some back-migration of cod individuals from the GoR to the MB also occurs, especially related to spawning (21), potentially stabilizing the source population and hence the source-sink dynamic (10).

According to our study, the potential for cod to reestablish in the GoR is related to processes operating in the MB. If the cod population in the MB recovers to high levels, its distribution range may expand northward, and potentially reinvade the GoR. For this to occur, enduring low fishing pressure and favorable hydrological conditions for cod in the source habitat are necessary. Under this potential scenario, and to aid cod persistence in the GoR and other marginal habitats, fishing mortality should be constrained at the local scale to remain well below the influx rate from the MB.

Resolving how and under which circumstances local systems respond to in situ processes vs. external forcing is crucial for understanding ecological dynamics in a metaecosystem context (1, 3). We have shown that extreme variations in a heavily exploited predator population, coupled with large variations in its distribution range, have cascading repercussions not only on its main distribution area (17, 33, 34) but also in adjacent systems, which usually are not under the trophic control of the top predator (for examples in terrestrial systems, see refs. 12 and 13). Therefore, high cod population levels can, through transitory spillover, link food webs that would otherwise be functionally nearly isolated. This finding provides empirical evidence that dispersal as a regional process may act to alter local predator–prey dynamics, prey competitive interactions, and food web structure (2). Our study also adds a metaecosystem perspective to the debate on the key role of top predators in structuring marine ecosystems (35).

Cross-system fluxes may play an important role in linking food-web dynamics across natural habitats, and investigations of their variations are crucial to forecast species' and ecosystems' responses to climate changes, fisheries, and eutrophication. The integration of landscape and food-web ecology is therefore central in the management of exploited resources and in ecosystem conservation.

Materials and Methods

Data on Cod Population from the Main Basin. Time series of cod biomass (ages 2+) in the Baltic MB at the start of the year was calculated with an extended survivors analysis (XSA) using commercial landings and scientific surveys from the MB. This is the standard methodology used within the International Council for the Exploration of the Sea (ICES) stock assessment framework (19). Maps of cod distribution were created using standardized catch per unit effort (catch per hour of trawling) data collected during the Bottom International Trawl Survey (36) and previous research surveys. Three distribution maps were produced, corresponding respectively to the periods before, during, and after the high cod population of the early 1980s.

Data from the Gulf of Riga. Data on cod commercial catches in the GoR were obtained from the former Latvian Fish Resources Agency. A fishery-independent index of cod biomass (catch per hour) in the GoR was calculated using the eelpout survey performed in the GoR by the same agency since 1974. Herring biomass and abundance at the start of the year were calculated using XSA stock assessment models (19). Time series of herring mean weight at age 3 was available from ICES (19). Weight at age 3 was used because it is the first age-class fully reproductive. Herring fishing pressure was estimated using the ratio of commercial catches to biomass (37). The zooplankton species used include the main copepods *Eurytemora affinis* and *Acartia* spp., and the indigenous cladocerans *Bosmina longispina maritima*, *Evadne nordmanni*, and *Podon* spp. These species constitute ∼90% of the zooplankton consumed by herring in the GoR (38). The invasive cladoceran *Cercopagis pengoi*, occurring in our samples since 1997, was not included because of its potential multiple effects on different trophic levels, which may confound the analyses and make the interpretation of the results difficult: *C. pengoi* is prey for herring and a predator of other zooplankton species, which are in turn prey for herring and predators of phytoplankton (39, 40). However, the structural changes in the GoR ecosystem started before the appearance of this plankter in our time series. Zooplankton data in the GoR were provided by the former Latvian Fish Resources Agency. Details on zooplankton sampling procedure and identification can be found in a previous work (41). Water temperature (°C) and salinity [practical salinity units (psu)] were averaged over the 0 to 50- and 0 to 20-m depth. Chl *a* (mg/m^3) was averaged over a 0 to 20-m depth and nutrients (mmol/m^3) over a 0 to 50-m depth. Runoff (m^3/s) from the Latvian main river Daugava was used as proxy for the nutrient load to the GoR (27).

Statistical Analysis. To analyze the effect of different predictors on each trophic level (response) of the GoR, we used GAMs (42). GAMs offer the main advantage of being able to model both linearity and nonlinearity between responses and predictors. The following additive formulation was used in the full models:

$$Response = a + s(V_i) + \ldots\ldots + s(V_n) + \varepsilon.$$

where a is the intercept, s the thin-plate smoothing spline function (43), $V_i \ldots V_n$ the predictors, and ε the random error.

The trophic levels considered were cod biomass index, herring abundance, and zooplankton and phytoplankton biomass (as indexed by Chl *a*) in GoR. We also analyzed the effects of the predictors on herring mean body size (weight at age 3) as proxy for individual growth rate. The predictors for each trophic level were selected based on acknowledged ecological and physiological mechanisms, and typically included top-down (predation driven), bottom-up (resource driven), and hydrological forcing (Table S3).

In the herring body size and zooplankton (prey of herring and thus influencing its growth) models, herring abundance, rather than biomass, was used as top-down predictor because biomass contains, by definition, a growth signal, making response (weight at age) and predictor (biomass) not independent a priori. All variables were log-transformed before analyses.

In the GAM modeling, we retained only the predictors that were statistically significant (final model) using a backward stepwise procedure. From the full model, the nonsignificant predictor with the lowest significance level was excluded at each step and the model run again. This procedure was repeated until all of the predictors were significant. We limited the maximum degrees of freedom acceptable for each term to $k = 4$. A Gaussian distribution with an identity function was used in almost all of the models because of the normal distribution of the response variables. For the cod biomass index, a quasi-Poisson distribution was used because of its larger flexibility in fitting the data distributions, especially for zero-inflated data. For the herring weight at age model, a gamma distribution was used. We calculated the deviance explained by the final models and the deviance contribution of each predictor selected in the final models. The deviance contribution of the predictors was estimated based on the percent difference in explained deviance of the final models after deletion of one predictor at a time while keeping all the rest (i.e. with replacement) (22). This procedure should give a good estimate of the explanatory power of each covariate as long as the estimated degrees of freedoms of the remaining covariates do not change largely, as was the case. The partial effects of the predictors were linear or quasilinear in all of the models (Figs. S3 and S5), and therefore the sign of the relationships was also provided. Model residuals were inspected using graphical methods (44).

For the lower trophic levels (i.e., plankton), the values of the predictors were taken the same year of the response variables, because we assumed that the high turnover rates of zooplankton and phytoplankton would make them respond promptly (the same year) to changes in the environment (17). For herring population size, the use of a different approach was needed due to the co-occurrence in the population of several cohorts persisting in time (years). Therefore, we constructed the predictors' time series to represent their potential influence on herring population over the period of existence of a cohort. Because the herring population consists mainly of eight age classes (from 1 to 7, plus the 8+ constituted by all of the ages older than 7 y pooled together) (19), herring abundance at time t is the result of forces that have acted over the previous 8 y (i.e., at years $t - 1$, $t - 2$, … , $t - 8$). However, because in average ∼90% of the herring population is constituted by fish 1–4 y old (19), we used predictors values only from $t - 1$ to $t - 4$ in this analysis, which decreased the influence of age classes scarcely represented in the herring population and the loss of too many data points for the analysis. The response (herring abundance) at time t, thus, were related to the 4-y mean (at time $t - 1$, … , $t - 4$) of each predictor. The same approach was used in the investigation of temperature effects on Baltic sprat landings 3 y after (45), and in the study of the main factors driving sprat population size in the Baltic Sea (17).

The statistical analyses were performed using the mgcv library of R 2.12.0 (http://www.r-project.org/). The significance level was set to $\alpha = 0.05$ for all tests.

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Effect of probe contact pressure on the photoplethysmographic assessment of conduit artery stiffness

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Abstract. Currently, photoplethysmography (PPG) is a frequently studied optical blood pulsation detection technique among biophotonic and biomedical researchers due to the fact that it shows high potential for estimating the arterial stiffness (AS). The extraction of diagnostically useful information requires standardized measurement procedure with good repeatability. However, the effects of a crucially important factor—the optimal contact pressure (CP) of the probe—are often ignored. Also, CP values are not reported to evaluate those effects. It is hypothesized that AS estimated from PPG pulse wave 2nd derivative parameter b/a is strongly inconsistent when recorded at nonoptimal probe CP. Our pilot study confirmed this during *in vivo* PPG recordings from conduit artery sites on five healthy subjects at variable probe CP (0 to 15 kPa) by using 880 nm reflectance type sensor, force transducer, and PPG alternating current (AC) signal pulse area derived optimal CP criterion. The b/a values, calculated from PPG with variable CP, showed variation >300 percent. In contrast, at the optimal CP, the b/a showed high repeatability (coefficient of variability <5 percent). The effect has been explained with exponential pulse pressure-volume relationship model which indicates the optimal CP range. [DOI: 10.1117/1.JBO.18.2.027004]

Keywords: arterial photoplethysmography; arterial stiffness; probe contact pressure; transmural pressure.

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1 Introduction

Inadequate alterations of arterial stiffness (AS) are known to be a timely, determinable indicator of endothelial dysfunction.[1] AS is a term widely used by clinicians to describe the elastic properties of the arterial wall and is directly proportional to the Peterson modulus.[1,2] Recent studies demonstrate that the stiffness of the conduit arteries is recognized as an important contributor to the development of cardiovascular disease as well as an independent predictor of cardiovascular morbidity and mortality, which includes hypertension and end-stage renal disease in general population.[3–5] The noninvasive assessment of AS consists of three main approaches, which include pulse wave velocity (PWV) measurement, pulse pressure or blood flow waveform analysis, and distensibility measurements of arterial pressure and diameter.[6] Photoplethysmography (PPG) is a simple, and promising, optical method for the stiffness evaluation using the signal pulse wave contour analysis and aforementioned PWV approaches even though the optical collection of reliable physiological information from the conduit arteries is still a challenging and controversial issue. There are a limited number of papers related to this technique.[7–10] The oldest, and most investigated, method for stiffness assessment is the PWV determination as it was suggested to be the gold standard.[11] However, this seemingly reliable method has many disadvantages such as the requirement of recording from two distant arterial sites, the lack of a precise definition to what constitutes the foot of the waveform and errors in the calculation of the path length between the optical probes. Moreover, PWV, itself, is sensitive to changes in heart rate (HR), blood pressure, and to the small changes in the arterial wall properties which may not be detected between individuals as the data generated can often show a considerable scatter for a given age range.[12–14] The other way to determine the optical measurement of AS is to use the pulse waveform derived parameters such as reflection and augmentation indexes. Still, many authors have a controversial opinion about the use of these indexes in the assessment of AS.[6,15] The promising, and comparatively new, AS characterizing index is the b/a ratio which is computed from the PPG AC pulse wave 2nd derivative peaks, a and b, as shown in Fig. 1. Proposed by Hashimoto et al., this index demonstrates a good correlation with AS changes altered by age, hypertension, and other vascular risk factors, and have been proven in many other studies.[16–19] However, there are reports of PWV and b/a indicating atherosclerotic alterations differently, yet providing valuable information concerning vascular modifications of aging.[20] Due to apparent simplicity of the measurement and commercially available equipment, the majority of published optical AS assessment studies shows the tendency to use the PPG by applying the probes on the fingertips and ear lobes, the diffuse and arterio-venous anastomoses rich vascular beds which are largely influenced by local temperature changes and sympathetic nervous system.[21–24] In contrary, few papers describe the procedure of AS assessment from superficial conduit arteries using PPG technique.[7,19] Hence, the lack of standardization in the PPG recording procedure and, particularly, in

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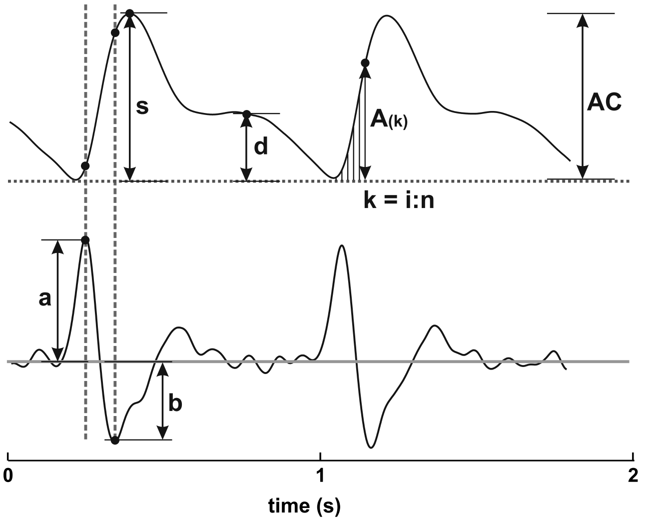

Fig. 1 PPG alternating current (AC) pulse waveform contour consisting of signal samples A_k; systolic peak amplitude s, diastolic peak amplitude d, 2nd derivative extremes a and b.

the unknown PPG probe CP conditions, may cause an inconsistency in the results when the pulse wave contour analysis derived parameters are computed.[25] In case of PWV, this has been explained by analyzing the tissue elastic properties beneath the PPG probe, similar to this study.[26] To the best of our knowledge, there were a few studies addressing the standardization issue and, currently, there are no papers suggesting any standardized criterion of the PPG probe CP except our previous pilot study results.[27,28] Current study focuses on development of methodology to achieve more reliable and valid PPG recordings. The aim of this study was to verify the effect of the PPG probe CP on the value of stiffness related parameter b/a and to clarify whether the previously developed optimal pressure parameter (OPP) can be used for a reliable recording of b/a.[28]

We hypothesized that the stiffness related PPG pulse wave parameter b/a is strongly inconsistent when recorded at nonoptimal probe CP.

2 Methodology

Five young and healthy subjects (3 male, 2 female, 23 ± 2 years old) were enrolled in this pilot study with their informed consent. This study was approved by the Scientific Research Ethics Committee of the University of Latvia, Institute of Experimental and Clinical Medicine. To perform measurement trials in resting conditions, subjects were held in a comfortable, supine position in a quiet and comfortable (23°C to 25°C) environment. The experimental setup and the design of PPG equipment were similar to those reported in our previous study revealing the OPP, as shown in Fig. 2.[28]

The PPG probe was placed on the skin over the three palpable pulse arterial sites (posterior tibial a., femoral a., popliteal a.), consecutively, in different recording trials, repeating the same procedure three times. The probe was fastened with the custom assembled micro-thread manipulator (UniSlide, Velmex Inc.) joined to film-type force transducer (FlexiForc A201, Tekscan) to provide variable CP recording.

Prior to the probe positioning, the arterial region (planned recording site) was insonated with an ultrasound system (Titan, Sonosite; L38 Linear array 10–5 MHz) by an experienced sonographer to reveal any abnormalities or peculiarities which might potentially interfere with a normal arterial site PPG recording as well as to measure the depth and diameter of artery. The stability of the systemic hemodynamic parameters, during the whole experiment, were confirmed by measuring the arterial blood pressure and heart rate by an oscillometric pressure monitor (UA-767Plus30, A&D Instruments) every 2 min. After the ultrasound examination and the determination of the location of the suitable arterial site by mechanical palpation, a single PPG probe was positioned on the skin over the conduit artery. During the recording, the probe CP was slowly increased to the maximum (which was determined by a complete disappearance of the PPG AC pulsations) or reduced until the probe lost contact with the skin. The data acquisitions, of both the PPG signal and force transducer signal, were performed simultaneously at a 4 kHz sample rate and analyzed offline with dedicated Matlab software "PPG Waveform Analysis" (Univ. of Latvia, IAPS, Rubins et al.). The stiffness related waveform parameter b/a was calculated from the 2nd derivative of PPG signal.

For indicating probe CP where PPG is being recorded in the conditions of unloaded arterial wall, OPP was calculated by Eq. (1) during signal processing in beat-per-beat manner:

$$\mathrm{OPP} = \frac{d}{s}\frac{1}{(n-1)}\sum_{k=1}^{n} A_k, \tag{1}$$

where d/s is the diastolic to systolic peak ratio of the PPG signal and $k = i{:}n$ are the samples of each PPG AC pulse; A_k is the amplitude of each sample of PPG AC signal, as shown in Fig. 1.

3 Results

All the subjects examined with the ultrasound imaging showed a normal geometry of the arterial tree at the PPG recording sites and systemic hemodynamic parameters were held constant during PPG measurement procedure (HR = 68 ± 5 BPM; systolic pressure (P_{sys}) 118 ± 8 mmHg; diastolic pressure (P_{dia}) 78 ± 5 mmHg). The depths and diameters of the arteries differed among the subjects and the measurement sites. The smallest diameter and depth were observed for the posterior tibial artery (diameter: 2.1 to 3.2 mm; depth 3.2 to 5.2 mm), the medium values were for the femoral artery (diameter: 6.1 to 8.1 mm; depth 10.4 to 30.2 mm), and the highest values for the popliteal artery (diameter: 7.7 to 9.1 mm; depth 8.6 to 20.5 mm). The literature confirmed our results while

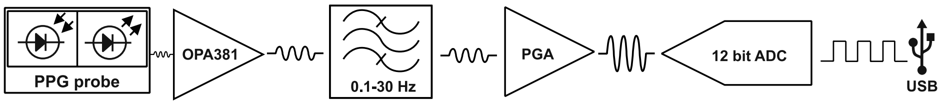

Fig. 2 Schematics of the PPG apparatus: a transimpedance amplifier (OPA381, Texas Instruments) based reflectance type PPG sensor with a photodiode (BPW34-FA, Osram, peak spectral response: 880 nm), an active 1st order feedback circuit 34 Hz low-pass filter, and the 875 nm LED (SIR91-21C/F7, Everlight, 400 mW, a transmission angle 20 deg, diameter 1.9 mm). The probe was connected to custom designed biosignal amplifier with an integrated band pass filter, consisting from 0.1 Hz 2nd order high-pass and 30 Hz 6th order low-pass filters.

indicating the difference in diameter between the genders and the difference of age.[29] The obtained PPG waveforms were typical for the particular arterial sites and were similar to those reported by Sapoznikov, Loukogeorgakis and our research group.[7,9,10] The optimal probe CP values significantly differ at each recording site ($p < 0.05$). Consequently, the highest optimal CP values were observed for the popliteal artery (15.2 ± 4.0 kPa), medium for the femoral artery (11.8 ± 2.9 kPa), and the lowest values for the posterior tibial artery (10.9 ± 3.1 kPa), mean ± sd. These results are in accordance with the ultrasound examination data in which they confirm the relationship of the arterial depth and the amount of underlying tissue.

Overall, we observed a 300 percent to 400 percent variation of the stiffness related parameter b/a during the incremental and decremental change of probe CP (states A, B, and C), as depicted in Figs. 3 and 4.

As expected, the b/a values obtained at the optimal probe CP showed a negligible measurement site dependency (CV < 6%), popliteal artery (0.66 ± 0.08), femoral artery (0.69 ± 0.09), and posterior tibial artery (0.73 ± 0.09), mean ± SD as shown in Fig. 5. Such data represents the b/a values corresponding to the conduit AS of young and healthy subjects that can be explained by a similar compliance of the muscular type arteries. Similar results are illustrated in another prominent study.[16] That can be explained by a similar compliance of the muscular type arteries.

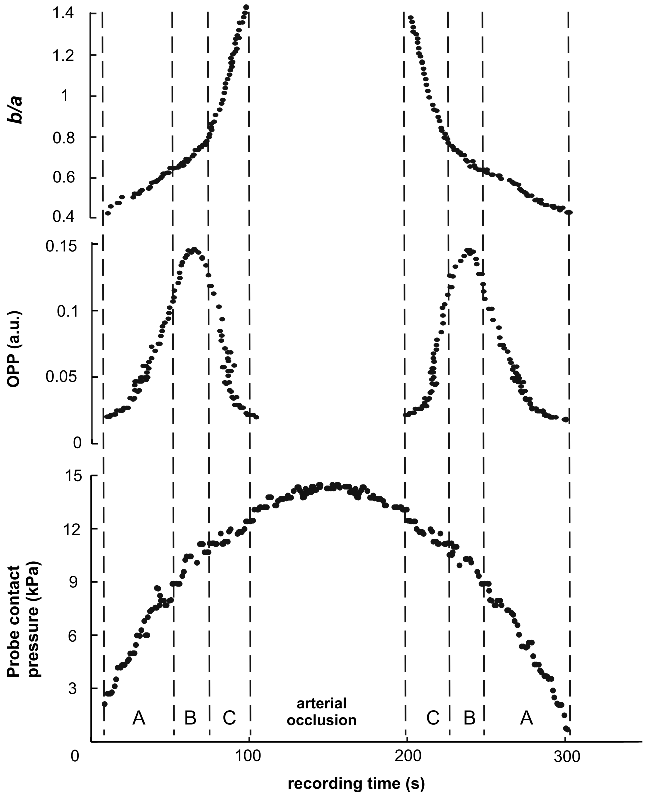

Fig. 3 Representative example of one subject: PPG recorded from the femoral site. The variable probe contact pressure (CP) (bottom curve) induced changes of the PPG AC waveform parameters: optimal pressure parameter (OPP) and 2nd derivative amplitude ratio b/a, which is related to arterial stiffness. The probe CP is partly conducted to the arterial wall. The OPP maximum indicates zero transmural pressure where the external pressure affecting the arterial wall P_{effect} is equal to the mean arterial pressure (MAP), state B, providing repeatable, standardized measurement conditions. It also indicates the conditions of the unloaded artery wall and optimal CP value. At state A probe CP is insufficient, intra-arterial pressure exceeds the external pressure and the transmural pressure is positive, while in state C transmural pressure is negative and the P_{effect} is between MAP and systolic pressure. The PPG pulse wave contours, corresponding to each measurement state (A, B and C), are illustrated in Fig. 4.

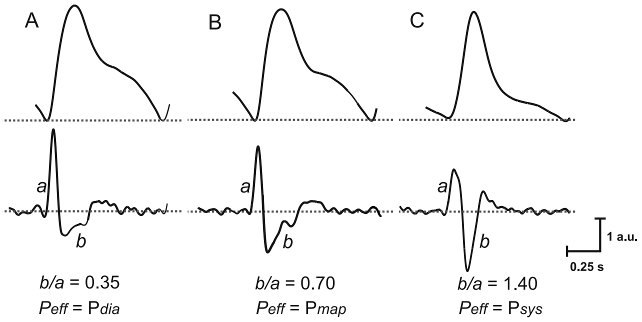

Fig. 4 PPG AC pulse wave and the corresponding 2nd derivative parameter b/a, recorded from one subject. States A, B, and C are in accordance with probe contact pressure condition states in Fig. 3, and P–V relationship in Fig. 6.

The difference between b/a values at states A and B is not significant ($p < 0.05$). A significant b/a error, compared to optimal CP conditions, arises only if the PPG signal is being recorded at conditions where P_{effect} is higher than the MAP, state C ($p < 0.001$; one way repeated measures analysis of variance). This is consistent with our own previous observations during measurements of arterial bed PPG (unpublished data).

4 Discussion

More detailed explanation of our results can be made by using the arterial P–V relationship model. According to the Marey's criterion, the OPP maximum indicates, on the PPG measurement conditions, where the arterial wall is unloaded (the P_{effect} equals to MAP and PPG AC pulse area reaches maximum value).[30] Instead of the mean PPG AC pulse area, we used area derived parameters, OPP, which additionally reflects the relationship between the systolic and diastolic wave of the PPG signal and is uncoupled from the DC component fluctuations. The probe CP influence, on b/a, could be explained by

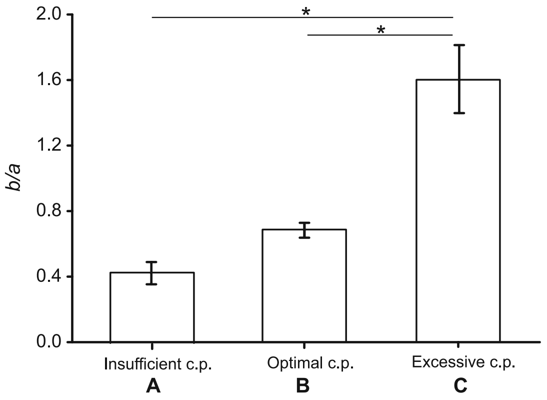

Fig. 5 Representative example of b/a values calculated from PPG signal from one subject, three measurement sites, at all three contact pressure (CP) states (A—insufficient CP; B—optimal CP, refers to maximum OPP; C—excessive CP, Fig. 3) expressed as mean ± sd. Significant difference is marked by asterisks ($*P < 0.05$).

the exponential model proposed by Raamat and Baker.[31,32] The model describes the arterial volume (V) dependence on the transmural pressure, P_{transm}, by the system of equations:

$$V = \begin{cases} V_{\text{bal}} e^{\frac{C_{\text{bal}}}{V_{\text{bal}}} P_{\text{transm}}} & for\ P_{\text{transm}} \leq 0 \\ V_{\text{sys}} - (V_{\text{sys}} - V_{\text{bal}}) e^{\frac{C_{\text{bal}}}{V_{\text{sys}} - V_{\text{bal}}} P_{\text{transm}}} & for\ P_{\text{transm}} \leq 0 \end{cases}, \tag{2}$$

where V_{bal} and C_{bal} are the arterial volume and compliance at the zero transmural pressure, while V_{sys} is the arterial volume at systolic pressure.

Numerous studies show the direct relationship between the oscillating arterial volume and the PPG signal AC waveform[33,34] and, therefore, from this, we associate the arterial P–V relationship with PPG pulse wave.

To explain the b/a dynamics near its peak values in Fig. 3, the PPG AC waveform should be examined by realizing the zero transmural pressure, $P_{\text{transm}} = 0$, at the upper limit state, systolic pressure, which is the highest pressure value within the pulse cycle. Then, the arterial wall is unloaded only at the peak of the pressure wave while, at all other times, it will be constricted by the excess pressure imposed by the state C in Figs. 3 and 6.

In the state of zero transmural pressure, arterial volume is always equal to V_0. During the state A, over one pulse period, oscillating volume of artery is above the level $V_0 = V_{\text{sys}}$ at C, thus, it is smaller than oscillating volume of artery during the state B which is in accordance to Marey's criterion of maximum oscillations at the MAP.

At the state C, the arterial compliance C_{bal} reaches its maximum value only at systole which produces the steeply rising PPG waveform and maximum b/a value, state C, at the Figs. 4 and 6.

During the optimal state B, the oscillating arterial volume, Fig. 6 crosswise, and the mean PPG AC area (and the OPP) reaches maximum, as shown in Fig. 3(b), which produces a more rounded and smooth waveform related to the zero transmural pressure at the MAP, as shown in Fig. 4(b).

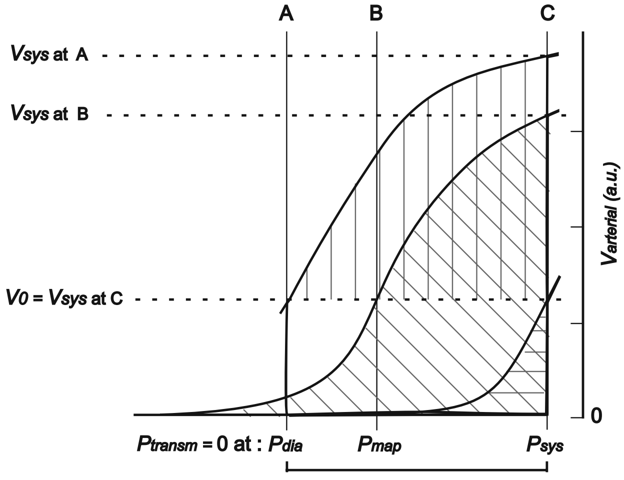

Fig. 6 P–V relationship at the different zero transmural pressure ($P_{\text{transm}} = P_{\text{arterial}} - P_{\text{effect}} = 0$) conditions. P_{transm} is zero at: P_{dia} (state A), MAP (state B) and P_{sys} (state C). Each state corresponds to its specific oscillating arterial volume (vertically, crosswise and horizontally hatched areas, respectively) producing PPG AC signal.

Hereby, the reliability of the OPP criterion was elegantly shown in our experiment during the incremental and decremental change of the probe CP, whereas, the b/a value returned within 5 percent tolerance between both cases. The same was observed for the optimal probe CP value. Being dependent on the measurement site, it returned within the same tolerance both incremental and decremental CP cases.

Overall, it indicates that our measurement is repeatable. Our suggestion to use the PPG waveform parameter, such as the OPP, is based mainly on the evidence of the close tolerance of repeated maximum values (usually $\text{CV} < 5\%$) every time when CP is optimal. Our observations show that even if the OPP amplitude maximum values vary more, the AS parameter b/a and probe CP values, returned from the analysis, are only slightly different ($\text{CV} < 8$ percent). This suggests that decreasing the probe CP from the maximum to the optimal is correct when the OPP reaches the next maximum instead of the value of the previous one. The variable probe CP induced notable changes to the PPG parameters OPP and b/a. Parameters display similar values during increment and decrement of probe CP within the measurement trial, thus, confirming the consistency of measurement conditions. This should be considered as the most important reason why the noninvasive contact-manner PPG measurements should be performed in the controlled probe CP conditions. Currently, this factor is still not properly acknowledged and often disregarded while designing the experimental protocol.

So far, OPP range is being calculated offline after the measurement trial, which allows only a fraction of PPG pulses that corresponds to its maximum value to be selected (selection criteria). By improving the data acquisition software with real-time OPP computation, initial OPP range assessment could be added to the experimental protocol prior to performing physiological measurements, thus, ensuring that signal is recorded in standardized conditions.

5 Conclusions

We conclude that, in the case of an uncontrolled probe contact pressure, PPG waveform derived parameters, particularly b/a, are inconsistent. Therefore, the results obtained in such measurement trials should be interpreted with precaution. Our present findings can be considered a step toward standardization of probe contact pressure and more reliable recording of contact-manner PPG signal.

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Monitoring seasonal changes in microbial populations of spruce forest soil of the Northern Temperate Zone

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Abstract. Soil microbial populations in the Northern Temperate Zone have been poorly studied in comparison with extreme environments. The aim of the work was to study the seasonal changes in the microbial populations of spruce forest soil of the Northern Temperate Zone using classical methods of microbiology and molecular biology. Upper horizons in two *Picea abies* stands on sod-podzolic and illuvial humus podzol soil were analysed. Sampling was done monthly over a period of twelve months (May 2009–April 2010). Microbial communities in both experimental plots showed different responses to the analysed environmental factors. In the sod-podzolic soil only the fungal DNA amount was significantly higher in the rest period (October–April) in comparison with the active vegetation period (May–September) and the number of *Penicillium* spp. colonies was larger in the active vegetation period. In the other soil the number of maltose utilizing bacteria, yeasts, and *Penicillium* spp. and other culturable filamentous fungi was significantly higher in the active vegetation period, while the fungal DNA amount was elevated in the rest period. Although ARDRA did not reveal differences, sequencing of 84 fungal isolates showed different compositions of the communities. Sørensen's index between the plots was low (0.29). Comparing the active vegetation period with the rest period, the index was higher (0.48). Although all tested fungal isolates from the rest period were able to grow at 4 °C, none of them showed psychrotrophic growth characters.

Key words: forest soil, filamentous fungi, active vegetation period, rest period, Shannon–Weaver diversity index, qPCR.

INTRODUCTION

Several studies are available about the impact of seasonal changes upon soil microorganisms in arctic soils (Nemergut et al., 2005), deserts, tropical soils, and other soils in extreme environments (Dion, 2008). Comparatively few investigations have been published about microbial populations in the soils of the Northern Temperate Zone (NTZ) in which climatic conditions during winter are characterized by permanent or temporal snow cover and a significant decrease of topsoil temperature below zero.

Functional diversity of bacterial populations was assessed in sandy soils over a climatic gradient in Western Canada using sole-carbon-source utilization analyses (Staddon et al., 1998). Response of soil bacteria and endomycorrhizal fungi to nitrogen deposition in northern forest ecosystems was investigated in north-western Lower Michigan (Waldrop et al., 2004). Studies on the seasonal dynamics of the microbial population of alpine dry meadow sites in the Colorado Rocky Mountains revealed that the microbial community undergoes a shift in function and genetic structure at the change of winter and summer seasons (Lipson et al., 2002). Microbial biomass and activity increase to maximal levels in late winter under the snow and then decline during snowmelt. This results in an increased availability of nitrogen, which is used by plants and the developing summer microbial community (Brooks et al., 1998; Lipson et al., 1999, 2000; Schmidt et al., 2004). Potential allelochemicals, such as simple phenolic compounds, are released from fresh litter in autumn and make up carbon and energy sources for the microbial community that develops in autumn and winter (Lipson et al., 2000, 2002). These potential allelochemicals are consumed before plant growth starts in spring (Schmidt & Lipson, 2004). In NTZ ecosystems litter decomposition (Zimov et al., 1993; Schmidt & Lipson, 2004) and gas accumulation (e.g., CO_2, CH_4, N_2O) take place under the winter snow packs. In the soil of jack pine (*Pinus banksiana*) stands in Western Canada Staddon et al. (1998) found significant positive correlations between the diversity of microbial populations and the soil pH, average daily temperature in July, mean annual temperature, degree-days above 18 °C, and degree-days above 5 °C.

The aim of our work was to analyse seasonal changes in the microbial populations in NTZ spruce (*Picea abies*) forest soil from two closely located forest stands with the same dominant tree species, but with different relief, soil, and vegetation types using classical methods of microbiology and molecular biology. The results confirmed that microbial populations are active under the snow during winter, while the two analysed soils showed different responses to environmental factors.

MATERIALS AND METHODS

Experimental plots

Two 40-years-old Norway spruce (*Picea abies* (L.) Karst.) stands in Malpils municipality (ca 30 km east of Riga, Latvia) were analysed. The plots were located ca 200 m from each other. Soil1 was sod-podzolic soil (Cutanic, Stagnic Albeluvisols) with *Oxalidosa* forest type. Soil2 was illuvial humus podzol (Placic, Rustic, Albic, Folic, Stagnic Podzols) with *Myrtilloso-polytrichosa* forest type (forest type classification according to Buss (1997), and soil types according to Latvian and FAO WRB (2006) soil classifications). Forest stands were heavily infected with the root rot fungus *Heterobasidion parviporum* (Grantina et al., 2010).

Soil1 was located at the upper part of a hill on moraine sediments and Soil2 in the depression of the relief on glaciofluvial sediments. The topography caused differences in the soil drainage between the experimental plots; the groundwater level was higher in Soil2. During winter only upper horizons of Soil1 were frozen (I–III). The snow cover of ca 0.50 m thickness lasted from January until mid-March.

Meteorological data

The raw meteorological data were obtained from the database of the Latvian Environment, Geology and Meteorology Centre, as recorded at the Meteorological Station in Riga. Average daily and monthly air temperature and total precipitation for every month were calculated.

Soil sampling and physico-chemical analysis

Soil samples for the microbiological analysis were taken in the middle of every month (9th–12th day, May 2009–April 2010) from 0–10 cm and 11–30 cm depths. Observations were divided into active vegetation period (AVP; May–September, average daily temperature $t > 10\,°\mathrm{C}$) and rest period (RP; October–April, $t < 10\,°\mathrm{C}$). A soil sampling plot was a circle with three transects on which nine samples (100 g) were taken at both depths at distances of 0.3 m, 1.0 m, and 3.0 m from the centre. Samples from the same depth were combined, resulting in three samples from both depths. Samples were placed in sterile plastic bags (*Nasco* WHIRL-PAK), stored at +4 °C for a few days until the plate count of culturable microorganisms was made, and after that stored at –20 °C. These samples were used also for the estimation of soil moisture content according to ISO 11465. For all other physico-chemical analyses soil samples (1–3 kg) were taken from each soil horizon in September 2009. Chemical analyses were made using (Expert Panel on Soil, 2006).

Analysis of culturable soil microorganisms

In order to estimate the number of colony forming units (CFU) of culturable filamentous fungi (CFF), yeasts, and maltose utilizing bacteria by a plate count method, soil sample dilutions were prepared by adding 10 g of soil to 90 mL of sterile distilled water. Suspensions were homogenized for 1 h on a horizontal shaker. After that serial dilutions were prepared, and 0.1 mL of dilutions 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were used. Agarised malt extract (MEA; 30 g/L, pH 5.5, from Biolife) was used as a growing medium that is favourable for fungi, yeasts, and certain groups of bacteria. Further in the text all these groups together are referred to as culturable microorganisms. The incubation time was 5 days and the temperature was $20 \pm 2\,°\mathrm{C}$. The CFU were expressed per gram of dry soil. Every soil sample was analysed in three replicates.

Genera of filamentous fungi were determined after 10 days of incubation according to morphological characteristics and light microscopy results.

Extraction of total soil DNA and quality control

Total soil DNA was extracted with a PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc.), which uses harsh lysis for DNA extraction. Samples (250 mg) were homogenized using a horizontal Mixer Mill Type MM 301 (Retsch) at a maximal speed of 30 Hz (1800 oscillations/minute) for 10 min. The amount and purity of the DNA were established spectrophotometrically using Ultrospec 3100 Pro (Amersham Biosciences) at wavelengths of 230, 260, and 280 nm in order to determine the contamination with proteins and humic compounds (Yeates et al., 1998).

ARDRA of soil DNA

For the amplified ribosomal RNA gene analysis (ARDRA) the fungal rRNA gene region that contains two internal transcribed spacers (ITS) and the 5.8S rRNA gene (ITS1-5.8S-ITS2) was amplified with the primers ITS1F and ITS4 (Gardes & Bruns, 1993).

The reactions in Eppendorf Mastercycler Personal were carried out in 50 μL volume. The mixture contained 0.4 μL of Hot Start *Taq* DNA Polymerase; 5 μL of 10X Hot Start PCR Buffer; 5 μL of dNTP Mix, 2 mM each; 4 μL of 25 mM $MgCl_2$; 0.75 μL of Bovine Serum Albumin 20 mg/mL (all reagents from Fermentas); 1 μL of each 25 μM primer (OPERON Biotechnologies); 30.85 μL of sterile distilled water; and 1 μL of DNA template. The polymerase chain reaction (PCR) conditions were as follows: the initial denaturation step of 4 min at 95 °C, 40 s of denaturation at 95 °C, 40 s of annealing at 52 °C, 1 min of primer extension at 72 °C (30 cycles), and final extension 10 min at 72 °C.

For ARDRA analysis with restriction endonuclease *Bsu*RI (Chabrerie et al., 2003) the amplification products after the PCR were precipitated by 450 μL of 90% ethanol and 0.3 M sodium acetate (pH 5.0). The precipitated DNA was washed with 70% ethanol, air dried, dissolved in ddH2O, and digested with *Bsu*RI (Fermentas). The restriction products were visualized in 6% polyacrylamide gel electrophoresis using a Mighty Small^{TM} II (Hoefer) unit. Gels were photographed and analysed with KODAK1D software. For the calculation of the Shannon–Weaver diversity index (H') the following equation was used: $H' = -\sum p_j \log_2 p_j$, where $p_j =$ relative intensity of individual band (Gabor et al., 2003).

Quantitative PCR

In quantitative PCR (qPCR) the ITS1-5.8S-ITS2 rRNA gene region of higher fungi with primers ITS1F and ITS4 was used to determine the amount of total fungal DNA within the total soil DNA. Combined soil DNA samples for each soil depth were analysed in three replicates for every month's samples.

As both forest stands were infected with *H. parviporum*, we monitored with special interest the DNA amount of antagonistic fungi *Trichoderma* spp. of this tree pathogen (Korhonen & Stenlid, 1998). In order to estimate the amount of

Trichoderma spp. DNA, qPCR with the primers uTr and uTf (Hagn et al., 2007) was performed. Samples of every second month were used, each analysed in three replicates.

The reactions were carried out in 25 μL volume. The mixture contained 12.5 μL of Maxima^{TM} SYBR Green qPCR Master Mix 2x (Fermentas), 1 μL of each 25 μM primer, 9.5 μL of sterile distilled water, and 1 μL of the DNA template. The PCR conditions on 7300 Real time PCR system (Applied Biosystems) were as follows: initial denaturation 60 s at 95 °C; 40× (30 s of denaturation at 95 °C, 30 s of annealing at 55 °C for primers ITS1F and ITS4 or 60 °C for primers uTr and uTf, 30 s of primer extension at 72 °C). Calibration curves were built using serial dilutions of DNA from pure cultures of *Phlebiopsis gigantea* MSCL 702 and *Trichoderma harzianum* MSCL 309. In more detail the method is described in (Grantina et al., 2011).

Sequencing

A total of 84 isolates (45 sporulating, 39 sterile mycelia, i.e. not sporulating when kept at +4 °C for several months) representing dominant filamentous fungi were isolated from the plates used for the enumeration of CFF and subcultured on MEA. Genomic DNA from approximately 0.25 g of mycelia was extracted using the method developed by Cenis (1992). The extracted DNA was amplified in PCR with the primers ITS4 and ITS1F. Afterwards 5 μL of amplified products was subjected to a sequencing protocol with BigDye Terminator v. 3.1 Cycle Sequencing Kit with primer ITS1F. The sequencing of the samples was performed in the Latvian Biomedical Research and Study Center. The obtained sequences were analysed using Staden Package 1.6.0. and checked against the database of the National Centre for Biotechnology Information using the Basic Local Alignment Search Tool for identification.

From the data of the sequenced isolates Sørensen's community similarity index C_s was calculated using the following equation: $C_s = 2C/A + B$, in which A and B are the number of species in samples A and B (Soil1 and Soil2), respectively, and C is the number of species shared by the two samples (Izzo et al., 2006).

Screening for psychrotrophic and mesophilic fungi

For growth temperature tests 29 strains (19 from RP, 10 from AVP) were used. Colonies were incubated at 4±2 °C and at 20±2 °C on MEA. Growth (expressed as colony diameter) was assessed several times during 20 days and growth rate (expressed as mm/day) was calculated.

Statistical analysis

The *F*-test, *t*-test ($\alpha = 0.05$), and correlation analysis were made with Excel (Microsoft, USA). Multiple regression analysis of the results and cluster analysis of ARDRA fingerprints were performed with the R package (R Development Core Team, 2009).

RESULTS AND DISCUSSION

Results of the meteorological data analysis

The average air temperature and total monthly precipitation during the analysed period are given in Fig. 1. The highest average air temperature at the meteorological station in Riga was recorded in June (+16.68 °C), the lowest in January (–10.41 °C). The smallest amount of precipitation was recorded in May (9.70 mm) and the highest in June (136.80 mm).

Soil characterization

Data on the element content and total nitrogen content in each horizon of both soil profiles are listed in Table 1. Information about soil texture is given in Table 2. Certain differences in the element content between the profiles are caused by soil formation processes and later influence of geological sediments and topography: moraine contains more exchange cations; nitrogen accumulates in the lower parts of the relief.

In either experimental plot there were no statistically significant differences of soil moisture between sampling depths (Fig. 2). However, due to the landscape

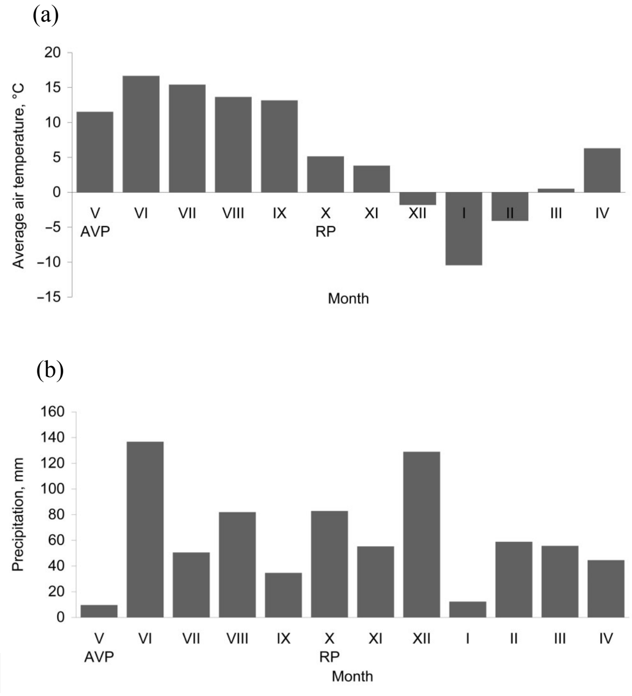

Fig. 1. Average air temperature (a) and total monthly precipitation (b) in the observation station at Riga during the analysed period. For the calculations raw meteorological data obtained from the database of the Latvian Environment, Geology and Meteorology Centre were used.

Table 1. Element content by soil horizons in the experimental plots

Horizon	Depth, cm	Ca, mg kg^{-1}	Mg, mg kg^{-1}	K, mg kg^{-1}	Na, mg kg^{-1}	P, mg kg^{-1}	N, %	pH$_{KCl}$
Soil1								
O	0–2	824.67±109.86	334.40±15.95	618.00±351.64	60.00±3.46	142.32±1.16	0.63±0.00	3.22±0.02
Ah	3–19	70.07±13.58	272.93±25.00	814.00±365.46	80.00±15.10	146.51±1.54	0.11±0.00	3.68±0.02
B	20–35	109.73±13.67	213.40±12.39	1140.67±49.57	70.00±10.00	103.13±1.34	0.06±0.00	3.98±0.01
EB	36–45	462.07±110.72	273.33±54.79	1318.00±3.46	98.00±10.58	63.76±0.77	0.06±0.00	3.56±0.01
Bt 1	46–81	2664.00±270.25	338.67±137.29	1320.00±3.46	140.67±51.47	76.69±0.59	0.00	3.95±0.00
Bt 2	82–130	2696.67±9.02	292.80±24.31	1282.00±10.39	125.33±23.44	125.80±1.32	0.00	4.62±0.02
Soil2								
O	0–11	2262.00±326.56	49.60±3.41	846.67±371.07	86.00±12.49	306.04±0.78	0.37±0.32	2.52±0.02
AhE	12–22	355.47±13.0	7.47±3.78	196.00±27.06	106.00±34.00	241.65±1.32	0.31±0.00	2.42±0.02
E	23–30	76.40±21.54	15.93±12.47	100.00±16.00	81.33±8.33	24.99±0.45	0.06±0.00	3.42±0.01
Bs 1	31–45	132.87±16.15	78.20±9.89	178.67±15.01	82.00±13.11	49.66±0.59	0.06±0.00	3.73±0.01
Bsg	46–61	223.53±53.53	247.07±33.62	182.00±7.21	71.33±11.02	72.09±0.97	0.06±0.00	3.88±0.01
Bs 2	62–102	310.60±4.85	230.80±17.17	200.00±19.29	76.00±16.37	82.96±0.77	0.00	4.04±0.02
R	103–135	409.47±100.82	252.73±30.66	249.33±36.90	54.00±3.46	111.29±1.38	0.00	4.08±0.01

Table 2. Soil granulometry by soil horizons in the experimental plots

Horizon	Clay, %	Silt, %	Sand, %	Characterization
Soil1				
O	Organic horizon			
Ah	10.9	24.7	64.4	Sandy loam
B	11.5	25.9	62.6	Sandy loam
EB	19.1	25.3	55.6	Sandy loam
Bt 1	22.5	26.7	50.8	Sandy clay loam
Bt 2	20.4	29.6	50	Loam
Soil2				
O	Organic horizon			
AhE	8.9	15.5	75.6	Loamy sand
E	3.8	5.2	91	Sand
Bs 1	6.2	2.8	90	Sand
Bsg	4.9	2.5	92.6	Sand
Bs 2	5.1	1.5	93.4	Sand
R	4.2	2.6	93.2	Sand

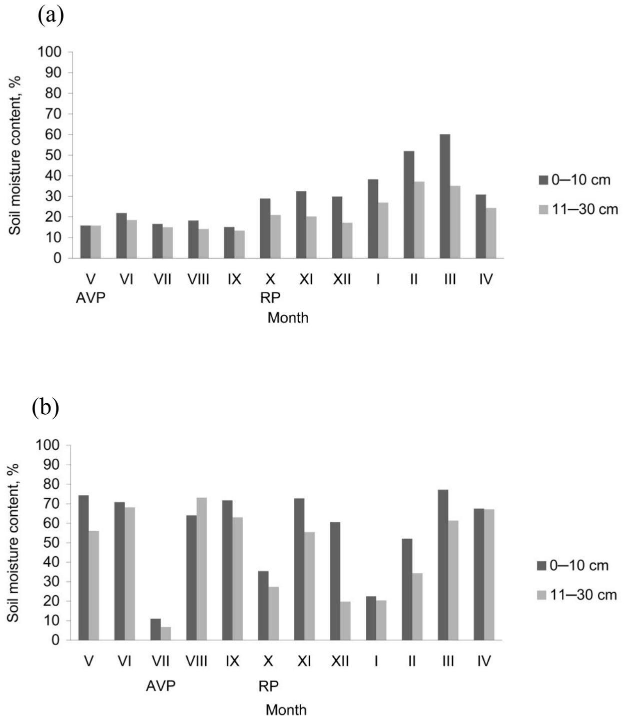

Fig. 2. Changes in soil moisture content during the analysed period in sampling plots Soil1 (a) and Soil2 (b) at two sampling depths (*n* = 1). Composite soil samples from each depth were used to estimate soil moisture content.

topography the moisture content was on average significantly higher in Soil2 ($F = 11.308$; $p < 0.001$). The topography of the plot and the presence of sphagnum moss caused ample fluctuations in its moisture content (6.69–72.55%). Still, in general there were no statistically significant differences of moisture content in Soil2 between the AVP and RP whereas in Soil1 the moisture content was significantly higher during the RP in comparison with the AVP ($F = 22.538$; $p < 0.001$).

The soil types and forest types of both analysed experimental plots are common in Latvia and in the NTZ. *Oxalidosa* and *Myrtilloso-polytrichosa* forests cover 19.5% and 3.6%, respectively, of the whole forest territory of Latvia. Sod-podzolic soil is the most common soil type in Latvia, but illuvial humus podzol is common only in lowlands (Karklins et al., 2009).

Soil chemical analysis showed that the soils of both experimental plots were very poor in nitrogen (0.63% in Soil1 and 0.37% in Soil2) in comparison with average values for the same forest types (1.8% in *Oxalidosa* forests and 1.5% in *Myrtilloso-polytrichosa* forests) in Latvia (Bardule et al., 2009).

The texture of Soil1 was typical of *Oxalidosa* forests, while Soil2 had a decreased content of clay and silt, but an increased content of sand in comparison with average soils of the *Myrtilloso-polytrichosa* forests in Latvia (Bardule et al., 2009).

Results of analysis of culturable microorganisms

Changes in the number of CFF in the experimental plots during the investigated period are presented in Fig. 3a, b. In Soil1 the numbers of obtained fungal colonies did not differ significantly between the AVP and RP and the colonies were equally distributed at both sampling depths (on average $(4.70 \pm 3.95) \times 10^4$ CFU g^{-1} dry soil). In Soil2 significant differences were observed between the two sampling depths ($F = 6.049$; $p < 0.05$) and between the AVP and RP ($F = 2.883$; $p < 0.05$; 2.15×10^5 and 1.84×10^4 CFU g^{-1} dry soil, respectively), accompanied by a remarkable variability within the 12 months; for example, low numbers of CFF were detected in July, October, January, and February.

The numbers of CFU of yeasts and maltose utilizing bacteria are shown in Fig. 3c, d. In Soil1 they were significantly different between the two sampling depths in the AVP ($F = 1.150$; $p = 0.020$; $(1.32 \pm 0.41) \times 10^6$ CFU g^{-1} dry soil at 0–10 cm depth, $(6.65 \pm 4.38) \times 10^5$ CFU g^{-1} dry soil at 11–30 cm depth), but similar at both depths in the RP. In general, differences in CFU numbers in Soil1 between the AVP and RP were not significant. In Soil2 the numbers of this group of soil microorganisms were not statistically significantly different between the two sampling depths but differed significantly ($F = 68.048$; $p = 0.032$) between the AVP and RP, being $(2.28 \pm 1.88) \times 10^6$ CFU g^{-1} dry soil on average and $(3.62 \pm 3.51) \times 10^5$ CFU g^{-1} dry soil, respectively.

So on average bacterial and fungal populations were in higher numbers in the AVP only in Soil2. Higher microscopic counts of bacteria in summer were recorded also by Lipson et al. (2002) in alpine dry meadow soils, but fungal

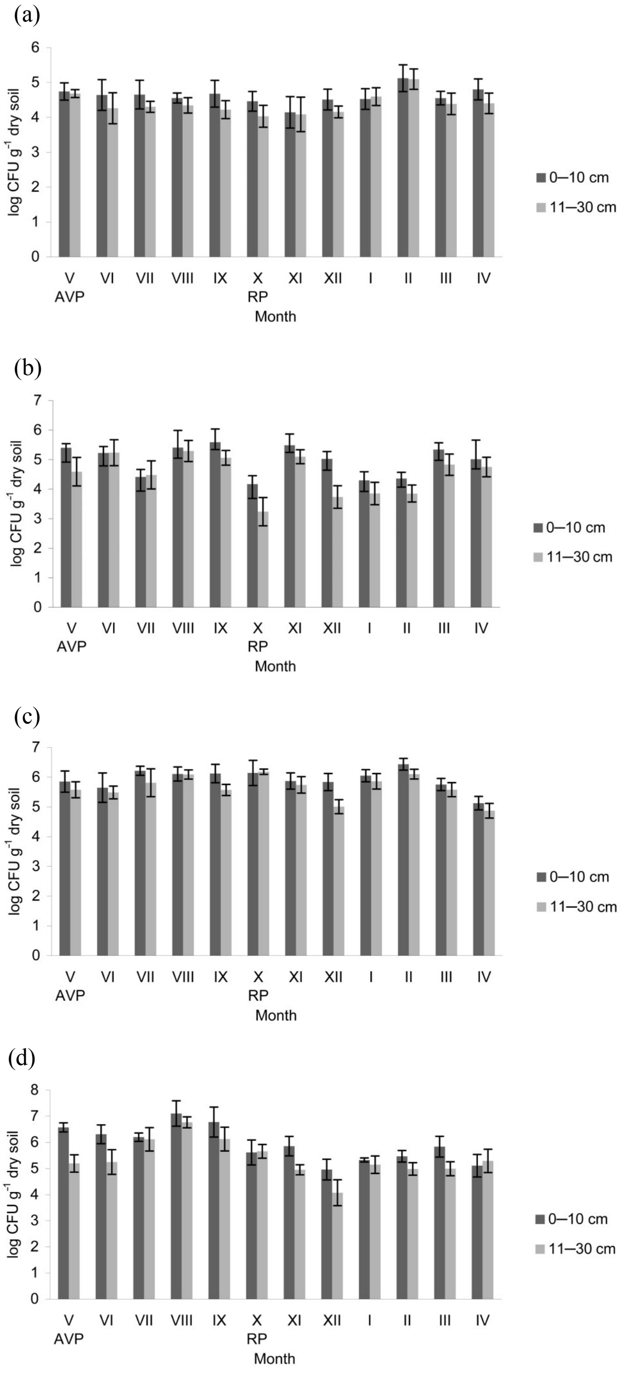

Fig. 3. Changes in the log number of CFF (a, b) and yeasts and maltose utilizing bacteria (c, d) during the investigated period in sampling plots Soil1 (a, c) and Soil2 (b, d) at two sampling depths (± SD; *n* = 9). Results were obtained after five days of incubation on MEA at the temperature of 20 ± 2 °C.

abundance expressed as length of hyphae (m g^{-1} soil) was increased in winter. These soils differ from our experimental plots in terms of vegetation and soil type, and they have at least two times higher nitrogen content in the A horizon (7.4 g N kg^{-1}). The authors concluded that winter microbial communities utilize more complex substrates such as cellulose but to a lesser extent simple compounds than do the summer communities.

As to particular dominant CFF genera on MEA, the percentage of *Penicillium* spp. was on average significantly higher during the AVP than during the RP in both plots: 33.20% versus 16.52% ($F = 2.407$; $p = 0.010$) in Soil1 and 38.36% versus 21.42% in Soil2 ($F = 2.415$; $p = 0.016$) (Fig. 4). Certain species such as *P. canescens* (one isolate) and *P. montanense* (four isolates) were detected only in the AVP, isolates of *P. spinulosum* (four isolates in total) were obtained in both periods according to sequencing results (Table 3).

Members of the genus *Mortierella* were evenly abundant in both the AVP and the RP in Soil1: 9.16% and 8.38%, respectively. In Soil2 the percentage of *Mortierella* spp. was low in the period July–January (on average 3.12%), but high in the periods May–June (29.93%) and February–April (24.71%). Looking at the particular species according to sequencing results, isolates of *M. alpina* (one isolate) and *M. gamsii* (one isolate) were detected only in the RP and *M. humilis* (one isolate) in the AVP, but isolates of *M. macrocystis* (four isolates in total) were detected in both periods.

The percentage of *Trichoderma* spp. was on average low in both plots, and at several sampling times *Trichoderma* spp. were not detected at all, e.g., in May–July and November–December in Soil1, and in October–November in Soil2. Detailed identification of fungal isolates using rRNA gene sequencing revealed that *Hypocrea viridescens* (March, one isolate) and *T. asperellum* (February, two isolates) were recovered only in the RP, but *H. pachybasioides* (six isolates in total) and *T. viride* (eight isolates in total) occurred both in the RP and AVP.

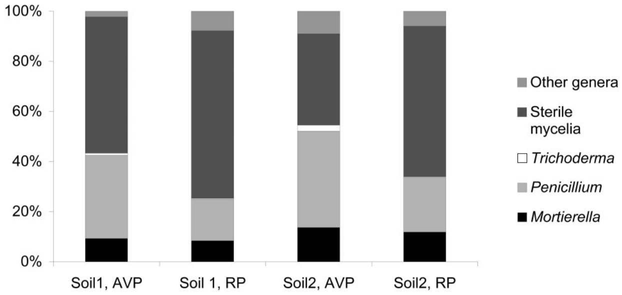

Fig. 4. Average percentage of the dominant CFF genera in the AVP and RP in the two sampling plots. Other genera include members from such genera as *Aspergillus*, *Botrytis*, *Cladosporium*, *Geomyces*, *Gilmaniella*, and *Mucor*. Results were obtained after ten days of incubation on malt extract agar at the temperature of 20 ± 2 °C.

Table 3. Fungal isolates of Soil1 and Soil2 identified by sequencing

Isolate[a]	CFU g^{-1} dry soil	Homolog sequence in NCBI	Max identity, %	Strain in MSCL[b]
Soil1, AVP				
Beauveria bassiana	10^5	GU566276.1	99	–
Beauveria geodes	10^4	U19037.1	98	968
Geomyces pannorum var. *asperulatus*	10^3	AJ938166.1	99	–
Hypocrea pachybasioides	10^3	FJ860796.1	100	–
Metarhizium anisopliae var. *anisopliae*	10^3	EU307900.1	99	950
Mortierella humilis	10^5	AJ878778.1	99	967
Mortierella macrocystis (1)	10^3	AJ878782.1	98	–
Mortierella macrocystis (2)	10^3	AJ878782.1	99	965
Mortierella sp. (1)	10^5	EU240040.1	99	–
Mortierella sp. (2)	10^4	EU240040.1	99	–
Penicillium canescens	10^5	DQ658168.1	100	–
Penicillium spinulosum	10^5	GU566247.1	99	–
Scytalidium lignicola	10^3	DQ093703.1	99	955
Trichocladium opacum	10^4	FN386299.1	100	958
Trichoderma viride	10^4	FJ481123.1	99	–
Umbelopsis ramanniana	10^3	DQ888724.1	99	–
Soil1, RP				
Beauveria bassiana	10^3	GU566276.1	99	960
Geomyces pannorum	10^3	FJ590611.1	99	947
Geomyces pannorum var. *asperulatus*	10^3	AJ938166.1	99	962
Geomyces vinaceus (1)	10^5	AJ608972.1	100	–
Geomyces vinaceus (2)	10^5	AJ608972.1	99	–
Hypocrea pachybasioides (1)	10^3	AY240841.1	99	–
Hypocrea pachybasioides (2)	10^4	AY240841.1	99	944
Hypocrea sp.	10^3	EU294196.1	99	–
Hypocrea viridescens	10^3	EU871022.1	99	–
Mortierella alpina	10^4	FJ161918.1	100	959
Mortierella gamsii	10^4	DQ093723.1	100	948
Mortierella macrocystis (1)	10^5	AJ878782.1	87	–
Mortierella macrocystis (2)	10^5	AJ878782.1	99	–
Mortierella sp. (1)	10^5	FJ810149.1	99	–
Mortierella sp. (2)	10^3	FJ810149.1	99	–
Mortierella sp. (3)	10^3	FJ810149.1	100	–
Mortierella sp. (4)	10^3	GQ302682.1	99	–
Paecilomyces carneus	10^5	AB258369.1	99	961
Penicillium sp.	10^5	FJ379821.1	99	–
Penicillium spinulosum	10^4	GU566247.1	99	–
Trichosporon porosum	10^5	AF414694.1	99	949
Umbelopsis ramanniana (1)	10^5	DQ888724.1	99	–
Umbelopsis ramanniana (2)	10^5	EU484205.1	97	941
Umbelopsis ramanniana (3)	10^4	DQ888724.1	98	–
Zygomycete	10^5	EF152531.1	90	–

Continued overleaf

Table 3. *Continued*

Isolate[a]	CFU g^{-1} dry soil	Homolog sequence in NCBI	Max identity, %	Strain in MSCL[b]
Soil2, AVP				
Beauveria geodes	10^3	U19037.1	99	–
Lecythophora mutabilis	10^4	HM036599.1	99	–
Mortierella sp.	10^4	FJ810149.1	99	–
Penicillium montanense (1)	10^5	AF527058.1	100	–
Penicillium montanense (2)	10^5	AF527058.1	100	–
Penicillium montanense (3)	10^4	AF527058.1	100	–
Penicillium montanense (4)	10^3	AF527058.1	100	942
Penicillium spinulosum	10^4	GU566247.1	99	–
Pezizomycete	10^5	GQ153212.1	82	–
Pochonia bulbillosa (1)	10^3	AB378551.1	99	–
Pochonia bulbillosa (2)	10^4	AB378551.1	99	964
Pseudeurotium bakeri	10^5	HM036612.1	100	–
Tolypocladium inflatum	10^5	GU354362.1	99	970
Trichoderma viride (1)	10^3	FJ872073.1	99	946
Trichoderma viride (2)	10^4	FJ481123.1	99	–
Umbelopsis isabellina	10^3	AJ876493.1	97	951
Soil2, RP				
Aspergillus cervinus	10^3	AY373845.1	98	963
Beauveria caledonica	10^3	AY532003.1	99	971
Geomyces pannorum var. *asperulatus*	10^3	AJ938166.1	99	–
Hypocrea pachybasioides (1)	10^4	AY240841.1	99	–
Hypocrea pachybasioides (2)	10^3	GU062213.1	98	–
Hypocrea pachybasioides (3)	10^3	AY240844.1	99	–
Lecythophora mutabilis	10^3	HM036599.1	99	956
Mortierella sp. (1)	10^4	EU240040.1	91	–
Mortierella sp. (2)	10^3	FJ810149.1	100	–
Mortierella sp. (3)	10^5	EU240039.1	99	–
Penicillium spinulosum	10^5	GU566247.1	99	952
Podospora appendiculata	10^3	AY999126.1	92	953
Pseudeurotium bakeri (1)	10^3	HM036612.1	99	–
Pseudeurotium bakeri (2)	10^3	HM036612.1	100	943
Rhizosphaera kalkhoffii	10^5	AY183366.1	100	954
Tolypocladium inflatum	10^4	GU354362.1	99	957
Trichoderma asperellum (1)	10^3	FJ605246.1	99	966
Trichoderma asperellum (2)	10^3	FJ605246.1	99	–
Trichoderma viride (1)	10^3	FJ481123.1	99	969
Trichoderma viride (2)	10^3	FJ481123.1	99	–
Trichoderma viride (3)	10^3	FJ481123.1	99	–
Trichoderma viride (4)	10^3	FJ481123.1	99	945
Trichoderma viride (5)	10^3	FJ481123.1	99	
Zygomycete (1)	10^5	AM292200.1	96	–
Zygomycete (2)	10^3	AM292200.1	96	–
Zygomycete (3)	10^3	AM292200.1	96	–
Zygomycete (4)	10^3	EF152547.1	92	–

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[a] Number in the parentheses indicates different isolates.
[b] Microbial Strain Collection of Latvia.

Several sequenced *Trichoderma* isolates were obtained as sterile mycelia on agar plates and did not sporulate until the end of the laboratory experiments in May 2010. Seasonality studies of *Trichoderma* species in spruce forest soil in Canada showed that members of this genus are more influenced by the competition among soil fungi than environmental factors such as soil moisture content (Widden & Abitbol, 1980). The small proportion of *Trichoderma* spp. in the soil fungal community can explain why trees in both stands were heavily infected with *H. parviporum*.

Representatives of the genus *Mucor* were present in a low percentage: on average 1–2% in Soil1, with the exception of the upper soil layer in May (9.69%), November (21.23%), and April (11.38%) when a larger proportion of this group of moulds was found among the isolates. In Soil2 *Mucor* spp. were more abundant in May–November (on average 9.28%) in comparison with December–April (1.28%). *Gilmaniella* spp., *Geomyces* spp., and *Aspergillus cervinus* were isolated only in the RP. *Botrytis* was isolated only in the AVP. Several insect pathogens were isolated from the analysed soils: *Beauveria bassiana* (RP, AVP), *B. caledonica* (RP), *B. geodes* (AVP), and *Metarhizium anisopliae* (AVP).

Table 3 gives an overview of 84 sequenced fungal isolates (45 sporulating, 39 sterile mycelia). The sequence database of the National Center for Biotechnology Information (NCBI) and Blast algorithm were used for sequence comparison; in most cases the sequence similarity exceeded 98%. One member of every species identified was deposited in the Microbial Strain Collection of Latvia.

Sequencing revealed different composition of the communities in Soil1 and Soil2: only five species were common in both experimental plots (*B. geodes*, *G. pannorum*, *H. pachybasioides*, *P. spinulosum*, and *T. viride*). From Soil1 19 unique species were isolated and identified, but in the case of Soil2 their number was 16. The corresponding Sørensen's community similarity index C_s was 0.29. In Soil1 six species were common in the AVP and RP (*B. bassiana*, *G. pannorum*, *H. pachybasioides*, *M. macrocystis*, *P. spinulosum*, and *U. ramanniana*). The corresponding C_s was 0.48. In Soil2 five species were common in both periods (*L. mutabilis*, *P. spinulosum*, *P. bakeri*, *T. inflatum*, *T. viride*, and *U. isabellina*). The corresponding C_s was 0.48.

Results of the screening for psychrotrophic and mesophilic fungi

Several fungal species isolated from the soil samples of the RP have been detected in arctic environments or recorded as growing at low temperatures (such as 0 °C): *G. vinaceus*, *G. pannorum*, *M. alpina* (Bergero et al., 1999), and also *T. porosum* (Danielson & Davey, 1973). Although all our isolates of these fungi were able to grow at 4 °C, their growth was not impeded at 20 °C either.

Several species were typically isolated only in the AVP; for example, *P. bulbillosa*, *S. lignicola*, *M. anisopliae* var. *anisopliae*, and *P. montanense*. From these species *M. anisopliae* var. *anisopliae* was not able to grow at low temperatures.

In laboratory conditions (in vitro) all the tested isolates showed mesophilic growth, none of them was psychrotrophic. The members of *Trichoderma*/*Hypocrea* had the highest growth rates among all strains at 20 °C and at 4 °C: 5.94–13.17 mm/day and 1.43–1.98 mm/day, respectively. Also the members of the genus *Mortierella* had high growth rates: 5.68 and 2.17 mm/day; an exception was the isolate of *Mortierella alpina* from the RP with slower growth rates: 2.24 and 0.85 mm/day. One isolate of *T. inflatum* from the RP and one isolate of the same species from the AVP showed identical growth rates at 20 °C and 4 °C: 1.41 ± 0.21 and 0.30 ± 0.09 mm/day, respectively. From all 29 isolates tested only *M. anisopliae* var. *anisopliae* was not able to grow at 4 °C. The isolate of *R. kalkhoffii* from the RP showed identical growth rates (0.57 ± 0.16 and 0.52 ± 0.09 mm/day) at both temperatures up to the 10th day of incubation, but later it started to grow significantly faster at 20 °C (1.08 ± 0.12 and 0.47 ± 0.06 mm/day). The comparative growth rate of the isolated strains in laboratory experiments is indicative. It may not reflect the actual growth rate of the same strains in soil (in situ), although the correlation between in vitro and in situ data is expected to be positive; for example, the intensive growth rate of *Trichoderma*, which was found in our experiments, is the basis for the use of these fungi in plant protection in situ.

Total soil DNA amount and results of qPCR

The success of the molecular methods depends on the quantity and quality of the extracted total soil DNA. Our results of the total DNA amount of 10.03 ± 4.29 $\mu g\ g^{-1}$ dry soil in Soil1 and 18.17 ± 9.59 $\mu g\ g^{-1}$ dry soil in Soil2 are similar or higher than those found in other investigations where MoBio kits have been used: 11.1 ± 0.3 $\mu g\ g^{-1}$ dry agricultural soil (Thakuria et al., 2008), 1.46 ± 3.7 $\mu g\ g^{-1}$ dry podzolic soil (Carrigg et al., 2007); 1.06 ± 0.35 $\mu g\ g^{-1}$ dry wetlands soil (Ning et al., 2009). Further molecular analysis of the DNA isolated from soil may be impeded by the presence of various inhibitors; many of them belong to humic compounds (Ning et al., 2009). We used Bovine Serum Albumin as a scavenger of potential inhibitors in the PCR reactions.

Changes in the amount of the total soil DNA in soil samples at the experimental plots are shown in Fig. 5a, b. The total soil DNA amount was similar at both sampling depths and in the RP and AVP, but it differed significantly ($F = 5.009$; $p < 0.01$) between the experimental plots: 10.03 ± 4.29 $\mu g\ g^{-1}$ dry soil in Soil1 and 18.17 ± 9.59 $\mu g\ g^{-1}$ dry soil in Soil2.

Changes in the amount of the fungal DNA during the analysed period are shown in Fig. 5c, d. No significant differences were observed at either analysed soil depth or in either experimental plot. However, in both experimental plots there were statistically significant differences between the AVP and RP. In Soil1 the fungal DNA amount was significantly ($F = 8.854$; $p = 0.013$) lower (8.08 ± 5.97 ng g^{-1} dry soil, excluding the value of the depth 10–30 cm in September) during the AVP than during the RP (112.40 ± 61.55 ng g^{-1} dry soil, excluding the

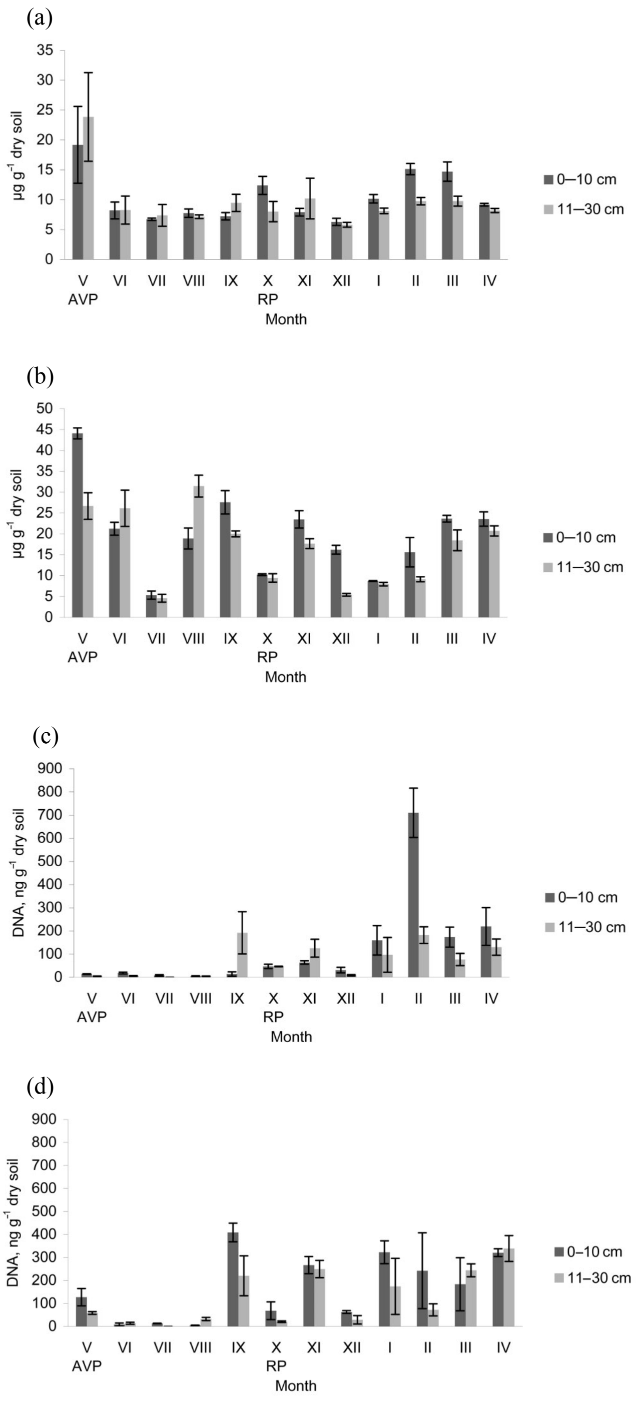

Fig. 5. Amount of total soil DNA (a, b) extracted with PowerSoil™ DNA Isolation Kit and total fungal DNA (c, d) estimated using qPCR in sampling plots Soil1 (a, c) and Soil2 (b, d) at two depths (±SD; *n* = 3) in the investigated period.

values of the upper soil layer in February and the lower soil layer in December). Similarly, the fungal DNA amount in Soil2 was significantly higher during the RP than during the AVP ($F = 1.331$; $p = 0.035$). During the RP the fungal DNA amount constituted on average 1.37% of the total soil DNA in Soil1 and 1.16% in Soil2. In the AVP the fungal DNA amount constituted a much lower proportion of the total soil DNA: on average 0.26% in Soil1 and 0.39% in Soil2. In both experimental plots the differences between the AVP and RP were statistically significant ($F = 3.125$; $p = 0.003$ and $F = 3.382$; $p = 0.003$).

The total amount of the fungal DNA increased at lower air temperatures in both experimental plots. This is in line with other investigations where it was determined that fungal populations dominate under the snow cover and that winter fungal populations are adapted to colder temperatures and more complex substrates (Lipson et al., 2002). It seems that cultivation independent methods such as fungal abundance in terms of hyphal length or molecular methods are more suitable for the studies of seasonal changes of fungal populations than the plate count method.

Our results agree with the observations that microbial biomass and activity increase to maximal levels in late winter under the snow and then decline during snowmelt due to the limitation of carbon sources (Brooks et al., 1998; Lipson et al., 1999, 2000; Schmidt et al., 2004). Our data demonstrated that during January and February when the soil was covered with snow the fungal DNA concentration values were higher than in the samples from March when snow was melting away. Also other analysed parameters showed a similar tendency. The best examples are the number of CFF in Soil1 in March (Fig. 3a), the number of CFU of yeasts and maltose utilizing bacteria in Soil1 (Fig. 3c), the total soil DNA amount in Soil1 (Fig. 5a), fungal DNA amount in Soil1 (Fig. 5d), and H' in both experimental plots (Fig. 6b) in March. This tendency is slightly different in Soil2, which remained unfrozen during the winter. In this experimental plot the values of the analysed indicators continued to increase also during March and decreased only in April.

The DNA amount of *Trichoderma* spp. was similar at both sampling depths in the experimental plots, and did not differ significantly between the AVP and RP (Fig. 6). However, it was significantly ($F = 32.088$; $p = 0.015$) higher in Soil2 (3.64 ± 3.10 ng g^{-1} dry soil) than in Soil1 (0.72 ± 0.44 ng g^{-1} dry soil, except the values in March). Both experimental plots had on average a higher percentage of *Trichoderma* spp DNA of the total fungal DNA in the AVP compared to the RP, but these differences were statistically not significant (6.68% and 4.50% versus 1.81% and 1.43% in Soil1 and Soil2, respectively). *Trichoderma* spp. DNA was found also in soil samples in which no CFU of *Trichoderma* genus were detected.

In general the correlation between the results of the conventional plate count (number of CFF) and the amount of fungal DNA was weak (correlation coefficient 0.18). This observation may reflect the accumulation of unculturable or psychrophilic fungi that enjoy optimum growth conditions under the snow

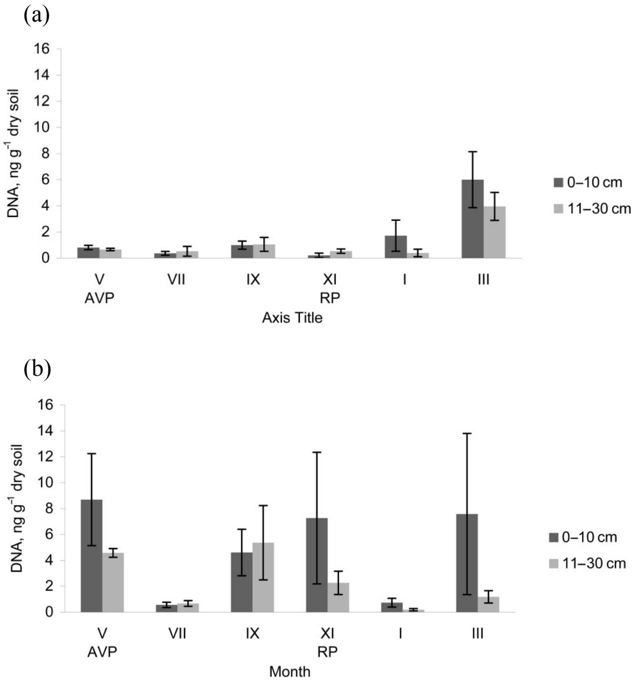

Fig. 6. Amount of *Trichoderma* spp. DNA estimated using qPCR in sampling plot Soil1 (a) and Soil2 (b) at both sampling depths (±SD; $n = 3$) in every second month.

cover in the presence of decaying plant litter. Their DNA could be amplified and identified, but we still could not grow the corresponding fungi in standard laboratory conditions.

Results of ARDRA

Typical results of ARDRA in 6% polyacrylamide gel electrophoresis are shown in Fig. 7 and changes of the Shannon–Weaver diversity indices of fungi in the experimental plots are given in Fig. 8. Fungal diversity indices were similar in both experimental plots and at both sampling depths. There were no statistically significant differences between the AVP and RP, but in some months (June, September, January) the fungal diversity was significantly lower than on average. The weak potential of molecular analysis derived diversity indices to evaluate the response of microbial populations to environmental factors has been recognized previously (Hartmann & Widmen, 2006). However, comparison of differences in the fungal community structure using cluster analysis (Fig. 9) showed tendencies for the soil samples from the RP to form a separate cluster in the case of Soil1 and several clusters in the case of Soil2. This means that the fungal communities were structurally different in the RP and AVP.

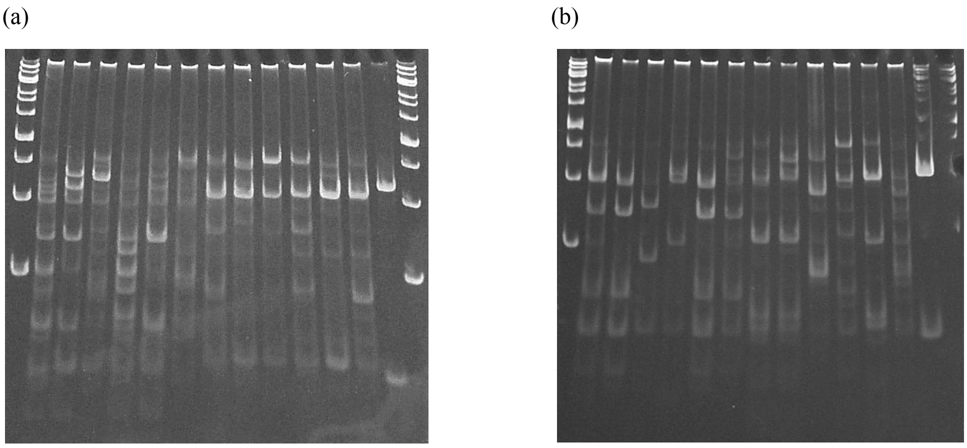

Fig. 7. Results of ARDRA in 6% polyacrylamide gel electrophoresis (August (a) and February (b)). Lanes 1–6 Soil1, 7–12 Soil2. +K, restriction products of *Heterobasidion parviporum* MSCL 981 DNA amplification. M, Gene Ruler 1 kb DNA Ladder (Fermentas).

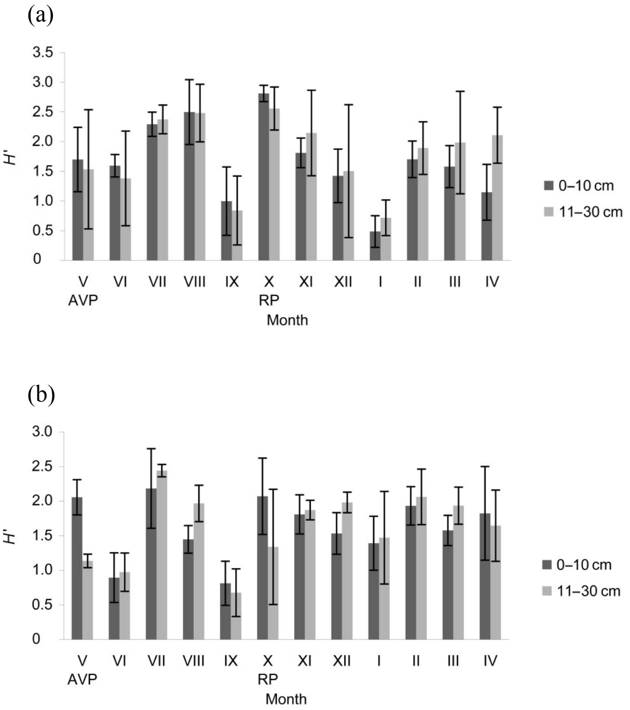

Fig. 8. Changes of the Shannon–Weaver diversity indices *H′* of fungi in sampling plots Soil1 (a) and Soil2 (b) at two depths (± SD; $n = 3$) in the investigated period. Indices were obtained using ARDRA in 6% polyacrylamide gels.

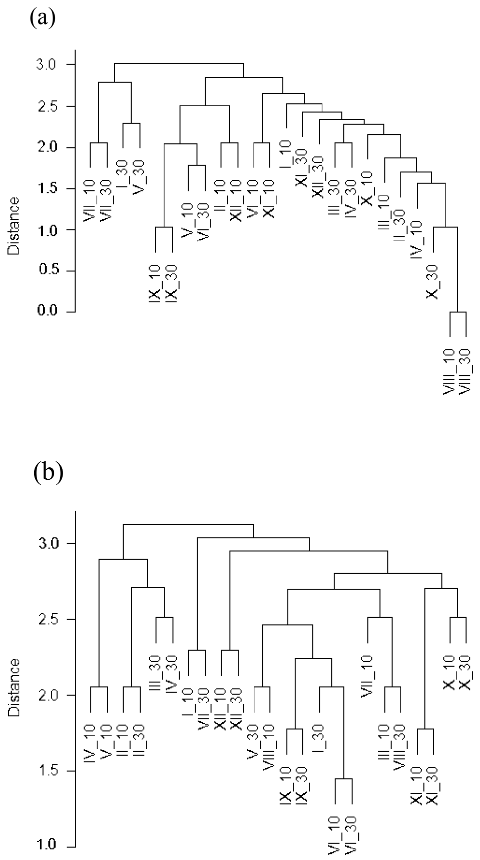

Fig. 9. Cluster analysis of seasonal fungal community structure differences in Soil1 (a) and Soil2 (b).

Results of the statistical analysis

In temperate regions the highest input of litter to the soil occurs in autumn when many plants are senescing and plant roots are dying. The winter microbial population starts to decompose the litter and to use phenolic carbon sources as well as to immobilize nitrogen. During snowmelt these populations die off and the accumulated nitrogen is used for plants and summer microbial populations. In cold systems the limiting factor for microbial growth is the availability of water but not the temperature. Under the snow even when the air temperature is significantly below 0 °C unfrozen water remains around the soil particles (Schmidt & Lipson, 2004).

Results of multiple regression analyses are listed in Table 4. According to these analyses, the number of CFF was significantly affected by soil moisture

Table 4. Results of multiple regression analyses

Factor	CFF	Yeasts and maltose utilizing bacteria	Total number of micro-organisms	Total amount of soil DNA	*H′*	Total amount of fungal DNA	Total amount of *Trichoderma* spp. DNA
Soil pH	NS	0.020[b]	0.005[b]	NS	NS	NS	NS
Average air temperature	0.01	NS	NS	0.020	NS	<0.001[b]	NS
Soil moisture content	<0.001	NS	NS	<0.001	NS	<0.001	<0.001
Multiple *R*-squared	0.387	0.459	0.489	0.551	–	0.537	0.627
p-Value of the model	<0.001	<0.001	<0.001	<0.001	–	<0.001	<0.001

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[b] – negative impact; NS, not significant; –, not applicable.

content (Pearson correlation coefficient $r = 0.59$; $p < 0.001$) and to some extent also by average air temperature ($r = 0.23$; $p = 0.01$). When both experimental plots were analysed separately, the data showed no correlation between air temperature and the number of CFF in Soil1, but in Soil2 there was a weak correlation ($r = 0.48$; $p < 0.001$). The significant positive impact of soil moisture content on the number of CFF explains the low number of fungal isolates from Soil2 in July, October, and January: during these months the soil in this experimental plot was very dry, the structure of the soil (Table 2) facilitated drainage and did not allow accumulation of the water precipitated during previous months.

According to the results of multiple regression analyses, the impact of soil moisture content and average air temperature on the number of yeasts and maltose utilizing bacteria and also on the total number of culturable microorganisms was significant, and high soil moisture together with high average air temperature can explain the increased numbers of CFU obtained in August. When the soil moisture content was higher, we observed higher numbers of CFF as well as a higher total amount of the fungal DNA, including *Trichoderma* spp. DNA. The numbers of yeasts and maltose utilizing bacteria were not so affected by soil moisture as the CFF, with the exception of the combination of high soil moisture with high air temperature as in the case of Soil2 in August.

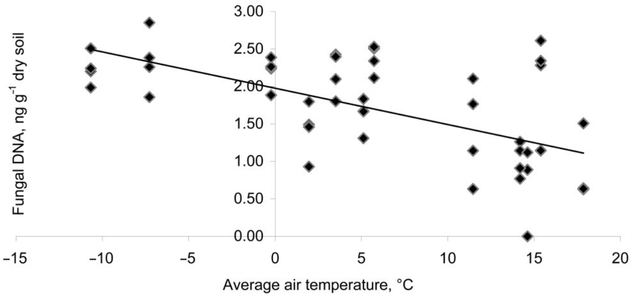
The soil pH varied in the analysed soil horizons from 2.42 to 3.68. In Soil2, which had a lower pH, the amount of yeasts and maltose utilizing bacteria was on average higher: 3.13×10^6 CFU g^{-1} of dry soil in the upper soil layer and 9.83×10^5 CFU g^{-1} of dry soil in the deeper soil layer versus 1.26×10^6 CFU g^{-1} of dry soil and 6.96×10^5 CFU g^{-1} of dry soil in Soil1. The soil pH, which was different between the experimental plots, significantly influenced the number of yeasts and maltose utilizing bacteria and the total number of soil microorganisms (data not shown). The total amount of soil DNA was influenced by the soil moisture ($r = 0.65$; $p < 0.001$) and average air temperature ($r = 0.22$; $p < 0.001$).

When data of the experimental plots were analysed separately, no correlation was found between the air temperature and soil moisture and the total amount of soil DNA in Soil1, while in Soil2 there was a weak correlation of the total amount of soil DNA with air temperature ($r = 0.42$; $p < 0.001$) and a moderately strong correlation with soil moisture ($r = 0.78$; $p < 0.001$). According to the results of *t*-test, these differences were not statistically significant, probably due to the fact that in Soil2 the CFU counts were significantly different between the AVP and RP.

Total amount of fungal DNA was significantly negatively affected by air temperature ($r = -0.60$; $p < 0.05$) (Fig. 10a) and positively affected by soil moisture content ($r = 0.44$; $p < 0.001$). Higher concentrations of fungal DNA at lower air temperatures were found in both experimental plots. The total amount of *Trichoderma* spp. DNA was affected only by soil moisture content ($r = 0.76$; $p < 0.001$) (Fig. 10b).

Fungal diversity expressed as the Shannon–Weaver diversity index H' was not influenced by any of the analysed factors.

(a)



(b)

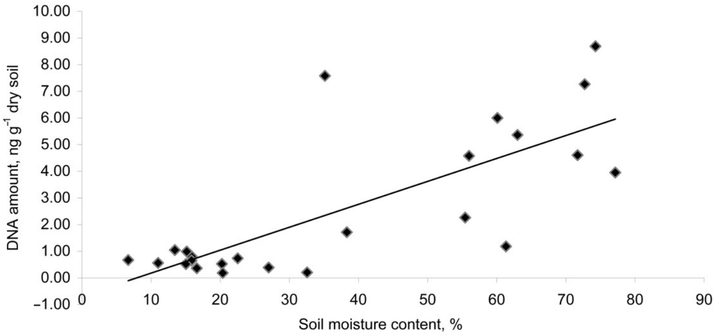


Fig. 10. Charts of correlations between average air temperature and total fungal DNA amount ($R^2 = 0.36$) (a) and between soil moisture content and *Trichoderma* spp. DNA amount ($R^2 = 0.58$) (b).

CONCLUSIONS

We can conclude that the microbial communities of both closely located experimental plots showed a different response to changing environmental factors during the AVP and RP. This can be explained by the fact that the air temperature and precipitation differed between the plots due to the differences in their microhabitat topography: soil type, texture, composition of chemical elements, which influenced the changes of soil moisture content and properties of soil horizons.

In the sod-podzolic soil (Soil1) only the fungal DNA amount varied significantly during the analysed period: it was significantly higher in the RP in comparison to the AVP.

In the illuvial humus podzol (Soil2) the number of culturable filamentous fungi, yeasts, and maltose utilizing bacteria as well as the abundance of *Penicillium* spp. were significantly higher in the AVP but the fungal DNA amount was significantly higher in the RP.

Several fungal species (*Geomyces vinaceus*, *G. pannorum*, *Mortierella alpina*, *Trichosporon porosum*) isolated from the soil samples of the RP have been detected in arctic environments or recorded as growing at low temperatures such as 0 °C. Although all our isolates of these fungi were able to grow at 4 °C, they did not show any psychrotrophic growth characters.

Several species were typically isolated only in the AVP (*Pochonia bulbillosa*, *Scytalidium lignicola*, *Metarhizium anisopliae* var. *anisopliae*, and *Penicillium montanense*). From these species *M. anisopliae* var. *anisopliae* was not able to grow at 4 °C. In general the growth rate was genus and species dependent.

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Sesoonsed muutused parasvöötme kuusemetsa mulla mikroobikoosluses

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Käesoleva uurimistöö eesmärgiks oli uurida sesoonseid muutusi parasvöötme kuusemetsa mulla mikroobikoosluses, kasutades traditsioonilisi ja molekulaarseid mikrobioloogilisi meetodeid. Materjal mikrobioloogilisteks analüüsideks koguti mulla kahest ülemisest horisondist kahelt eri tüüpi mullaga hariliku kuuse alalt 12 kuu pikkuse perioodi jooksul. Mulla mikroobikooslus oli seotud erinevate keskkonnateguritega uuritud aladel. Vegetatsiooniperioodiga (maist septembrini) võrreldes oli leetmulla korral seente DNA kogus mullas oluliselt suurem puhkeperioodil (oktoobrist aprillini) ja *Penicillium*'i perekonna liikide arv oli suurem vegetatsiooniperioodil. Huumusilluviaalse leedemulla puhul oli pärmide, *Penicillium*'i perekonna liikide ja kultiveeritavate niitjate seente arvukus suurem vegetatsiooniperioodil. 84 seeneisolaadi ITS-i järjestuste analüüs näitas kahe uuritud ala seenekoosluse suurt erinevust (Sørenseni indeks 0,29), kuid sesoonne erinevus oli väiksem (Sørenseni indeks 0,48). Puhkeperioodil kogutud seeneisolaadid olid võimelised kasvama 4 °C juures, kuid ükski neist isolaatidest ei olnud psührotroofne tüvi.

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ENVIRONMENTAL BIOTECHNOLOGY

Critical tests for determination of microbiological quality and biological activity in commercial vermicompost samples of different origins

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Abstract The aim of the present paper was to show that differences in biological activity among commercially produced vermicompost samples can be found by using a relatively simple test system consisting of microorganism tests on six microbiological media and soilless seedling growth tests with four vegetable crop species. Significant differences in biological properties among analyzed samples were evident both at the level of microbial load as well as plant growth-affecting activity. These differences were mostly manufacturer- and feedstock-associated, but also resulted from storage conditions of vermicompost samples. A mature vermicompost sample that was produced from sewage sludge still contained considerable number of *Escherichia coli*. Samples from all producers contained several potentially pathogenic fungal species such as *Aspergillus fumigatus*, *Pseudallescheria boidii*, *Pseudallescheria fimeti*, *Pseudallescheria minutispora*, *Scedosporium apiospermum*, *Scedosporium prolificans*, *Scopulariopsis brevicaulis*, *Stachybotrys chartarum*, *Geotrichum* spp., *Aphanoascus terreus*, and *Doratomyces columnaris*. In addition, samples from all producers contained plant growth-promoting fungi from the genera *Trichoderma* and *Mortierella*. The described system can be useful both for functional studies aiming at understanding of factors affecting quality characteristics of vermicompost preparations and for routine testing of microbiological quality and biological activity of organic waste-derived composts and vermicomposts.

Keywords Plant growth-affecting activity · Microbiological quality · Potentially pathogenic microorganisms · Vegetable seedlings · Vermicompost · Waste-derived compost

Introduction

The use of vermicompost in agricultural practice is continuously increasing. Vermicompost preparations are especially valuable in organic farming and in nutrient-poor soils as an important source of mineral nutrients (Gopinath et al. 2008). Other beneficial effects of vermicompost include soil conditioning (Kaur et al. 2010), enhancement of availability of mineral nutrients (Marinari et al. 2000), plant protection against pathogens and herbivores (Singh et al. 2003; Zaller 2006; Edwards et al. 2010), etc. In addition, chemical constituents of vermicompost produced by a concerted action of microorganisms and earthworms can have direct hormone-like effects on plants (Krishnamoorthy and Vajranabhaiah 1986; Tomati et al. 1988). However, some vermicompost preparations can be highly growth- and germination-inhibiting for several cultivated plant species, especially, at high doses (Warman and AngLopez 2010; Ievinsh 2011). Thus, the germination percentage of radish, marigold, and upland cress was observed to be significantly inhibited even by 12.5 % vermicompost extract or 5 % vermicompost soil substitution (Warman and AngLopez 2010). Similarly, substrate substitution with increasing doses of vermicompost (10 to 100 %) esulted in a linear decrease of germination percentage and inhibition of shoot growth of beetroot, bean, radish, pea, cabbage, and Swedish turnip seedlings (Ievinsh 2011).

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Consequently, biological activity of vermicompost at the level of potential plant growth-affecting capacity needs to be considered.

The wide variety of organic waste available as feedstock in vermicomposting represents a rich source of microbial diversity. Vermicompost can contain plant growth-promoting (free-living nitrogen fixers and nitrifying bacteria) and plant disease-protective microorganisms, like the fungi *Trichoderma* spp. (Gopal et al. 2009). Application of vermicompost can thereby be used to increase the level of potentially favorable soil microorganisms such as nitrogen fixers (Kale et al. 1992). It has been shown in previous studies that addition of vermicompost significantly increased the microbial activity in commercial substrates and was correlated with increased chlorophyll content of marigold leaves, shoot dry weight of tomato plants (Atiyeh et al. 2000), and other growth parameters of tomato seedlings (Atiyeh et al. 2001). Based on molecular analysis, it was found that microbial diversity and species composition of vermicompost samples were similar to those of vermicompost extracts produced from them. For example, the saprophytic bacteria *Sphingobacterium* and *Actinomyces* and ammonium-oxidizing bacteria *Nitrosovibrio* and *Nitrosospira* were found in both vermicompost and subsequent extracts (Fritz et al. 2012).

Evidently, vermicompost-associated microorganisms can affect humans during processing and handling similarly as in conventional composting (Deportes et al. 1995). For example, in a study in Italy of fungal populations of vermicompost produced from 70 % dung (from cows, poultry, and various zoo animals) and 30 % plant debris from various sources, it was found that the fungal populations were dominated by two species *Pseudallescheria boydii* and *Aspergillus fumigatus* (Anastasi et al. 2005). Both species are potential human and animal pathogens. *Aspergillus fumigatus* has also been found in aerosols of a conventional waste composting plant (Bru-Adan et al. 2009).

It has been shown that the level of artificially inoculated potentially harmful microorganisms such as *Escherichia coli*, *Enterococcus* spp., and *Salmonella* spp. in biosolids is significantly reduced due to the activity of earthworms already after 6 days of vermicomposting (Eastman et al. 2001). Selective reduction of pathogenic bacteria was observed in the vermicomposting of cow manure: The level of fecal enterococci, fecal coliforms, and *E. coli* was reduced but the level of *Clostridium*, total coliforms, and *Enterobacteria* remained unchanged (Aira et al. 2011). There is also some evidence that the level of potentially pathogenic fungi may remain unchanged (Beffa et al. 1998).

Much research has been conducted with general bacterial populations, and it is known that particular production conditions (feedstock, time, and method of vermicomposting) result in similar species composition of bacterial populations of vermicompost samples if the same earthworm species is used. For example, the average similarity coefficient among various products was nearly 80 % when estimated by comparable methods (Fernández-Gómez et al. 2012). However, there are no such studies regarding general fungal populations.

From a practical point of view, microbiological quality and biological activity of vermicompost preparations need to be quantified and compared in order to assure production of vermicompost with beneficial rather than harmful effects. Surprisingly, no comparative study of these parameters using a relatively wide range of different vermicompost samples has been performed so far. As various vermicompost preparations can contain different amounts of inorganic nutrients, these differences can mask plant growth-affecting activity in substrate substitution experiments. Also, plant response to vermicompost application is clearly genotype-specific (Bachman and Metzger 2008; Ievinsh 2011). Therefore, growth-affecting activity of vermicomposts needs to be compared using a soilless system with multiple crop species that differ in potential response to vermicompost-contained biologically active substances.

The aim of the present paper was to show that differences in microbiological quality and biological activity among commercially produced vermicompost samples can be found by using a relatively simple test system consisting of microorganism tests on six microbiological media and soilless seedling growth tests with four vegetable crop species. Specific concern raised was the possible negative effect of vermicompost on plants and its role as a source of pathogenic microorganisms (both for humans and plants). For comparison, two substrates from which vermicompost was subsequently produced were tested also.

Materials and methods

Vermicompost and compost samples and plants

Vermicompost and compost samples used in the present study were provided by four manufacturers in Latvia and labeled with letters A to N (Table 1). As test vegetable crop species, beetroot (*Beta vulgaris* L.) cv. Cylindra (Kurzemes Sēklas, Latvia), Swedish turnip (*Brassica napus* var. *napobrassica* L.) cv. Golden Ball (Sèklos, Lithuania), carrot (*Daucus carota* L.) cv. Nantes 4 (Kurzemes Sēklas, Latvia), and tomato (*Lycopersicon esculentum* L.) cv. Marmande (Kurzemes Sēklas, Latvia) were used. These species showed different responses to vermicompost application in a previous study (Ievinsh 2011).

pH and moisture content

Vermicompost and compost moisture content was determined according to the ISO 11465:1993 standard method

Table 1 Characteristics of vermicompost and compost samples used in the present study

Sample[a]	Characteristics	Manufacturer	Moisture content (%)	pH of sample (U)	pH of extract (U)
A	Vermicompost from cow manure, stored dry for 1.5 years at room temperature	1st SL Eko	6.4	6.87±0.01	7.44±0.02
B	Vermicompost from cow manure, stored wet for 1 year at 4 °C	1st	64.4	7.26±0.01	7.49±0.03
C	The same as B, stored dry for 1 year at room temperature	1st	5.8	6.85±0.03	7.23±0.03
D	Composted cow manure	2nd Mālpils Biotehnoloģijas Centrs	65.0	8.33±0.02	8.96±0.02
E	Vermicompost from composted cow manure, non-sieved	2nd	60.0	7.96±0.02	8.26±0.01
F	Sieved fraction (<2 mm) from E	2nd	52.0	7.55±0.02	7.93±0.02
G	Vermicompost from tree leaves and organic waste	3rd Foetida	64.0	7.89±0.01	8.38±0.03
H	Vermicompost from tree leaves and cow manure	3rd	55.5	8.55±0.03	8.53±0.02
I	Vermicompost from starchless potato pulp and composted grass	4th GAHA	53.7	7.68±0.02	7.38±0.02
J	Granulated form of vermicompost from starchless potato pulp and composted grass (thermal treatment at 40 °C applied during processing)	4th	21.7	7.96±0.02	7.68±0.01
K	Composted sewage sludge	3rd	82.2	6.26±0.01	7.06±0.01
L	Immature vermicompost from K and sawdust with tree leaves (initial phase)	3rd	64.3	8.01±0.03	7.88±0.01
M	Immature vermicompost from K and sawdust with tree leaves (medium phase)	3rd	62.2	7.51±0.02	7.95±0.03
N	Mature vermicompost from K and sawdust with tree leaves	3rd	61.7	6.65±0.02	7.37±0.03

[a] Each sample represents a different preparation otherwise indicated

(ISO 1993). pH was measured in distilled water in mass/volume ratio of 1:5.

Preparation of vermicompost and compost extracts

Vermicompost and compost samples were equilibrated with tap water up to relative moisture content of 65 % when necessary, with stirring for 6 h at room temperature. Samples were then mixed with water in the ratio 1:1 (*v*/*v*) and incubated at room temperature for 4 h. In the case of sewage sludge compost, proportionally less water was used. The resulting slurry was filtered through a paper filter and used as 50 % extract. By mixing of 50 % extract with tap water (1:4, *v*/*v*), diluted 10 % extract was prepared. This extract may contain lower concentrations of soluble and suspended content compared to an extract directly produced in appropriate ratio. Extracts were labeled A to N, following the sample nomenclature in Table 1.

Seedling growth tests

Seed samples were imbibed for 4 h in water (control) or in appropriate vermicompost or compost extract with 10 and 50 % concentration as described previously (Ievinsh 2011). Batches of 30 seeds were placed in Petri dishes on a filter paper soaked in the extract. Five Petri dishes with each extract were used for each crop species. Petri dishes were incubated in darkness at 23±2 °C for 6 days, and then the height of hypocotyl and the length of radicle were measured.

Overall plant growth-affecting activity of vermicompost or compost samples was characterized by summed stimulating and inhibiting activity. Percentages of both stimulation and inhibition for each compost or vermicompost sample were calculated for all four species tested (both for hypocotyl and radicle) at both concentrations used and summed separately as stimulating or inhibiting activity.

Analysis of cultivable microorganisms

Vermicompost and compost samples (10 g) were added to 90 ml sterile distilled water. Two replicates were made. The suspensions were homogenized 1 h on a horizontal shaker. Then, serial dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} were prepared from each replicate. To estimate the number of colony forming units (CFU) of cultivable filamentous fungi and bacteria by a plate count method, 0.1 ml of the dilutions was used. Total number of bacteria was estimated after 3 days of incubation on agarised tryptic soy broth (TSA; Biolife Italiana S.r.l., Milan, Italy) at 20±2 °C. Total number of cultivable filamentous fungi was estimated after 5 days of incubation on Rose Bengal agar with chloramphenicol (RBA; Biolife Italiana S.r.l., Milan, Italy) at 20±2 °C. Numbers of *E. coli* and coliforms were estimated after 1-day incubation on Endo agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 37±2 °C. Number of enterococci was determined after 1-day incubation on Enterococcosel agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 37±2 °C. Number of potentially pathogenic fungi was estimated after 5 days of incubation on Mycosel agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 37±2 °C. For the detection of the presence of *Salmonella* spp., 25-g samples were enriched in peptone water for 1 day at 37±2 °C followed by isolation on *Salmonella–Shigella* agar (S.S. Agar; Bio-Rad, Hercules, CA, USA) at 37±2 °C for 1 day. Identification of *E. coli* and *Salmonella* spp. was based on colony morphology after sample incubation on Endo or S.S. agar, respectively. Colonies with characteristic morphology from S.S. agar were subcultured on TSA and identified with the BBL CRYSTAL™ (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) test system. Number of cultivable microorganisms was expressed on dry mass (DM) basis.

Genera of cultivable filamentous fungi were determined after 10 days of incubation on Rose Bengal and Mycosel agar and after subculturing on malt extract agar (Biolife Italiana S.r.l., Milan, Italy) according to morphological characteristics and light microscopy results. In total, 64 dominant isolates of cultivable filamentous fungi from all compost and vermicompost samples were identified by means of molecular methods. Genomic DNA was extracted from approximately 0.25 g of mycelia using the method developed by Cenis (1992). Extracted DNA was amplified by PCR with primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). The PCR reactions in Eppendorf (Hamburg, Germany) Mastercycler Personal were carried out in 25 μl volume. The mixture contained 0.2 μl Hot Start *Taq* DNA Polymerase, 2.5 μl 10× Hot Start PCR Buffer, 2.5 μl dNTP Mix, 2 mM each, 2 μl 25 mM $MgCl_2$, 0.38 μl bovine serum albumin 20 $mg ml^{-1}$ (all reagents from Fermentas, Vilnius, Lithuania), 0.5 μl of each 25 μM primer (Operon Biotechnologies, Köln, Germany), 15.42 μl sterile distilled water, and 1 μl of DNA template. The PCR conditions were as follows: the initial denaturation step of 4 min at 95 °C, 40 s of denaturation at 95 °C, 40 s of annealing at 52 °C, 1 min of primer extension at 72 °C (30 cycles), and final extension 10 min at 72 °C.

Amplified DNA fragments from fungal isolates were sequenced by Macrogen Europe (Amsterdam, the Netherlands). Homology search of obtained sequences was performed against the National Centre for Biotechnology Information GenBank nucleotide database using the Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov). The sequences were submitted to GenBank (respective accession numbers KC461500–KC461563). All fungal strains were deposited in the Microbial Strain Collection of Latvia (Riga, Latvia).

Statistical analysis

Significance of differences between means was determined by the Tukey–Kramer test at the $\alpha=0.05$ level. In order to determine factors affecting microbial abundance and the effect of abundance of dominant fungal genera on stimulating activity, correlation analysis was performed with Excel (Microsoft, Redmond, WA, USA). Significance was evaluated at $p<0.05$ level. Both Pearson correlation coefficients (r) and determination coefficients (R^2) were determined.

Cluster analysis with maximal distance and stepwise multiple regression analysis were conducted using the R package (R Development Core Team 2009). The significance of effect of the following variables on summed stimulating or inhibiting activity was tested in the models: moisture content (in percent), pH of the extract, number of fungal genera, and log-transformed data of other microbial variables (total number of bacteria, total number of coliforms, total number of cultivable filamentous fungi, number of potentially pathogenic fungi).

Results

Seedling growth-affecting activity of vermicompost and compost extracts

In general, vermicompost and compost extracts at the employed lower concentration (10 %) stimulated hypocotyl growth of the tested species, except for carrot seedlings where both stimulating and inhibiting effects were found (Table 2). Similarly, radicle growth of the tested seedlings was stimulated by 10 % vermicompost extract treatment, although at a lesser extent than for hypocotyl growth. Radicle growth of tomato seedlings showed both inhibition and stimulation by 10 % vermicompost extracts.

Stimulating effects on hypocotyl growth by 10 % extracts made from vermicompost samples varied from 27 to 145 % for beet seedlings, 88 to 198 % for Swedish turnip seedlings, and 24 to 146 % for tomato seedlings (Table 2). For carrot seedlings, 10 % vermicompost extracts had effects ranging from 30 % inhibition to 49 % stimulation. Composted cow manure extract at 10 % concentration had no significant effect on hypocotyl growth of beet, carrot and tomato seedlings, but stimulated growth of Swedish turnip hypocotyls by 96 %.

Table 2 Effect of different vermicompost and compost sample extracts on height of hypocotyl and length of radicle of beetroot, Swedish turnip, carrot, and tomato seedlings

Sample[a]	Concentration (%)	Hypocotyl height (%)[b]				Radicle length (%)[b]			
		Rootbeet	Swedish turnip	Carrot	Tomato	Rootbeet	Swedish turnip	Carrot	Tomato
Control	0	100±8 b	100±12 ab	100±6 e	100±12 c	100±11 c	100±16 cd	100±7 d	100±15 de
A	10	208±26 hi	240±2 h	110±7 f	196±4 ij	177±9 g	105±10 cd	102±10 d	108±5 e
	50	137±6 de	185±7 e	37±9 bc	25±9 a	121±15 d	80±9 ab	41±11 a	21±7 a
B	10	220±10 i	285±5 i	95±13 e	209±11 j	214±10 h	120±12 d	124±15 e	149±19 gh
	50	189±21 gh	279±13 i	99±6 e	160±12 h	111±16 cd	167±7 f	115±27 de	118±13 ef
C	10	186±18 gh	216±20 g	137±5 g	246±13	100±16 c	101±6 c	113±4 de	106±5 e
	50	265±10 k	231±14 gh	34±4 b	130±14 ef	127±10 d	84±9 b	61±3 b	62±4 c
D	10	105±12 b	196±11 ef	100±4 e	108±10 c	137±16 ef	196±22 f	196±12 i	99±6
	50	84±4 a	146±22 cd	31±1 b	37±6 a	116±20 cd	66±6 a	42±2 a	39±2 b
E	10	133±25 d	188±4 de	80±10 d	124±6 e	93±14 c	144±1 e	114±14 de	79±12 cd
	50	117±11 bc	219±8 g	42±9 c	61±6 b	75±10 ab	79±7 a	58±2 b	56±5 c
F	10	127±12 c	222±7 g	70±4 d	126±18 e	168±10 fg	149±3 e	113±6 de	75±4 cd
	50	137±21 de	197±26 ef	25±2 a	114±8 de	71±7 ab	87±3 bc	38±13 a	58±10 c
G	10	185±18 gh	220±1 g	83±12 d	136±6 fg	179±24 g	149±21 e	161±11 gh	83±2 d
	50	161±2 f	244±3 h	30±6 b	157±11 h	64±11 a	102±6 c	71±11 c	54±2 c
H	10	164±20 fg	206±9 fg	70±4 d	117±12 de	149±20 ef	172±12 f	136±12 ef	94±7 d
	50	167±3 f	219±12 g	34±5 b	146±7 g	133±16 de	76±6 a	75±0 c	62±4 c
I	10	187±5 g	298±23 i	119±6 f	242±10 k	159±20 f	117±26 d	129±23 e	167±3 h
	50	210±6 hi	246±11 h	29±3 b	57±4 b	80±6 b	76±1 a	51±4 ab	54±15 bc
J	10	245±10 j	243±20 h	149±23 g	212±18 j	227±34 h	118±14 d	111±6 d	121±7 f
	50	245±24 jk	235±7 h	111±15 f	107±14 cd	136±4 e	66±4 a	76±2 c	75±3 cd
K	10	118±1 c	112±5 b	106±11 ef	97±5 c	162±14 fg	133±15 de	162±8 gh	122±0 f
	50	127±4 c	98±3 a	101±11 ef	103±7 c	109±6 c	129±20 d	117±11 de	99±5 de
L	10	125±1 c	119±7 b	114±7 f	111±7 cd	163±6 f	148±22 e	160±4 g	130±5 fg
	50	127±1 c	173±5 d	94±11 e	148±9 g	100±9 c	132±8 de	145±15 f	101±4 de
M	10	141±12 e	176±7 de	148±5 g	168±5 h	156±3 f	168±8 f	187±9 hi	134±5 g
	50	144±7 e	204±6 fg	114±7 f	162±10 h	83±3 b	101±6 c	134±6 e	64±5 c
N	10	130±6 d	141±4 c	159±6 h	150±4 gh	147±14 ef	151±12 ef	172±10 h	115±9 ef
	50	147±34 ef	205±10 fg	143±6 g	182±8 i	74±11 ab	118±9 d	149±17 fg	74±22 cd

[a] Abbreviations of sample names as in Table 1

[b] Data are means±SE from five independent measurements with 30 seedlings each. Means within the crop species with the same letter are not significantly different from each other (Tukey–Kramer test, $P<0.05$)

Regarding radicle growth, 10 % extracts of vermicompost samples had variable stimulating effects for beet, Swedish turnip, and carrot seedlings, ranging between 0 and 127, 1 and 96, and 2 and 96 %, respectively (Table 2). For tomato seedlings, the effects ranged from 21 % radicle growth inhibition to 67 % stimulation. Cow manure compost extract (10 %) stimulated radicle growth of beet seedlings by 37 % and of Swedish turnip and carrot seedlings, both by 96 %. However, radicle growth of tomato seedlings was not significantly affected by 10 % cow manure extract.

At higher vermicompost extract concentration (50 %), stimulation of hypocotyl growth was found only for beet and Swedish turnip seedlings, ranging from 27 to 145 and 88 to 198 %, respectively (Table 2). In contrast, growth of carrot hypocotyls was suppressed by all 50 % vermicompost extracts, with an effect ranging from 63 to 75 %, except for the B and J extracts that had no significant effects. However, both stimulation (from 7 to 60 %) and inhibition (from 43 to 75 %) were evident for 50 % extract-treated tomato seedling hypocotyls. Composted cow manure extract at 50 % concentration had no significant effect on hypocotyl growth of beet seedlings, a stimulating effect on Swedish turnip seedlings by 46 %, and inhibiting effects on both carrot and tomato seedlings by 69 and 63 %, respectively.

Radicle growth was only moderately inhibited by 50 % vermicompost extract treatment for Swedish turnip seedlings (from 16 to 34 %), except for extract G that had no significant effect, and extract B, which had a stimulating effect by 67 % (Table 2). All tested vermicompost extracts except B significantly inhibited radicle growth of both carrot and tomato seedlings, with the effect ranging from 24 to 62 % and from 25 to 79 %, respectively. The extract from vermicompost sample B significantly stimulated radicle growth in both carrot and tomato seedlings by 15 and 18 %, respectively. Radicle growth of beet seedlings was either significantly stimulated (from 11 to 36 %) or inhibited (from 20 to 36 %) by 50 % vermicompost extracts. Composted cow manure extract (50 %) significantly inhibited radicle growth of Swedish turnip (34 %), carrot (58 %), and tomato (61 %) seedlings and stimulated that of beet seedlings (16 %).

It was interesting to test the effect of storage conditions on growth-affecting activity of vermicompost extracts. Two vermicompost samples tested in the present system were stored dry in laboratory conditions for at least 1 year. No significant effect on radicle growth of vermicompost extract made from the two dry-stored samples (A and C) at 10 % concentration was evident for all tested vegetable species except for an effect of extract A on beet seedlings (Table 2). However, 10 % extract prepared from the same vermicompost material as C, only stored in wet refrigerated conditions (extract B), had a significantly high stimulating effect on radicle growth. In contrast, extracts made from dry-stored samples (A and C) had relatively high activity in stimulating hypocotyl growth, which was comparable to extract B made from wet-stored material.

Another question of interest was to test differences in quality of a material used for earthworm feeding affected plant growth-related activity of vermicompost preparations. Extracts made from vermicompost samples G and H produced by the same manufacturer had extremely similar growth-affecting activities in respect to both hypocotyl and radicle growth in spite of differences in feedstock (organic waste vs. cow manure).

It was also tested whether differences in biological activity might be due to various manipulations with vermicompost. Extracts made from non-sieved (E) and sieved (F) vermicompost samples produced by the same manufacturer showed highly similar activities in respect to hypocotyl and radicle growth, except for no effect of a 10 % extract E on radicle growth of beet seedlings (Table 2). Similar activities of extracts prepared from conventional (I) and granulated (J) vermicompost samples in respect to stimulation vs. inhibition of particular test species and organ were also found, but there were differences in the degree of the effect, sometimes being statistically significant. However, no general pattern in the observed differences was found.

A separate experiment was performed to evaluate the plant growth-affecting activity of composted sewage sludge in relation to vermicompost samples prepared from it in different degrees of maturity (samples K to N, Table 2). Extracts from sewage sludge had only negligible activity on hypocotyl growth, with a tendency to increase during vermicompost maturation. For Swedish turnip, carrot, and tomato seedlings, these effects were statistically significant. In contrast, tests on radicle growth showed no statistically significant differences in respect to the degree of vermicompost maturity.

Based on seedling growth tests, overall stimulating and inhibiting effects on plant growth of vermicompost and compost samples were calculated and compared (Fig. 1). When sewage sludge compost and respective vermicompost samples were excluded, stimulating effects ranged from 1,060 % (sample B) to 339 % (sample E), while inhibitory activities ranged from 10 % (sample B) to 276 % (sample F). In general, vermicompost extracts that showed higher stimulating activities showed lower inhibiting activities (R^2=0.87). Summed activities were similar for vermicompost samples produced from the same manufacturer, except for samples A to C (dry-stored), which had relatively lower stimulating activities and higher inhibiting activities.

Summed plant growth-affecting activities in extracts of sewage sludge compost (sample K) and vermicompost samples of different maturities (samples L to N) showed increase of stimulating activities from a low to medium level, accompanied by a relatively small increase of inhibiting activities (Fig. 1).

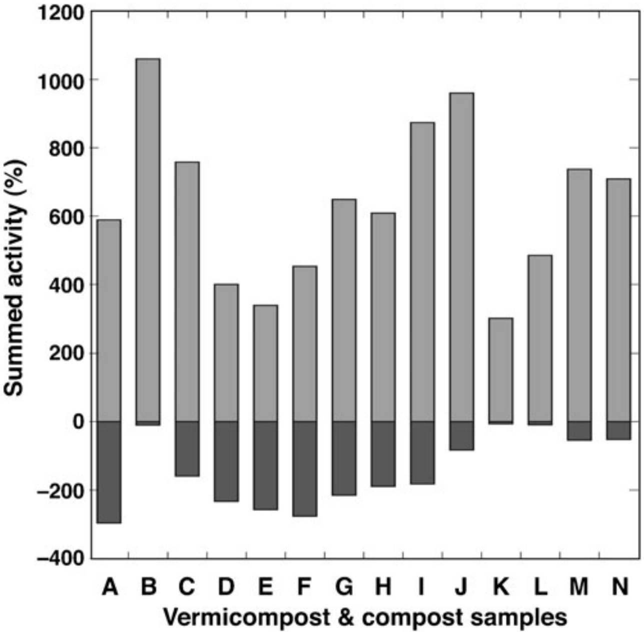

Fig. 1 Summed plant growth-affecting activity of vermicompost and compost sample extracts. Data are derived from the results shown in Table 2. Respective percent of stimulation (the same for inhibition, separately) for all vegetable crop species tested (both hypocotyl and radicle) was summed (eight entries together). Abbreviations of sample names as in Table 1

Abundance and diversity of cultivable microorganisms

The estimated counts of cultivable microorganisms on six microbial media are shown in Fig. 2a–d. There was a clear difference in the levels of almost all examined groups of microorganisms in completely dry (A, C) and medium dry vermicompost (J) samples, compared with relatively wet vermicompost samples. In dry vermicompost samples, CFU of all cultivable microorganisms were several-fold lower than in samples with high moisture content. For example, comparing samples A and C with B, the total number of fungi and potentially pathogenic fungi were lower by two- to 14-fold ($p=0.04$ and $p=0.0003$, respectively). The level of bacteria was eight- to nine-fold lower ($p<0.0001$), and the level of total coliforms was lower by even 14- to 35-fold ($p=0.006$) in samples A and C compared with B. The highest total number of bacteria among the all analyzed samples was in medium dry sample J ($p=0.005$), which was even higher than the level of bacteria in sewage sludge (K).

The indicators of fecal contamination, bacteria *E. coli* and enterococci, were detected in sewage sludge (sample K) and in two consecutive immature vermicompost samples (L and M; Fig. 3). *E. coli* was present also in mature vermicompost (sample N) at a level of $(1.66\pm0.67)\times10^{3}\,\mathrm{CFU\,g^{-1}}$ DM. *Salmonella* spp. were absent in all examined vermicompost and substrate samples. Colonies with morphology characteristic for *Salmonella* spp. from S.S. agar were identified as

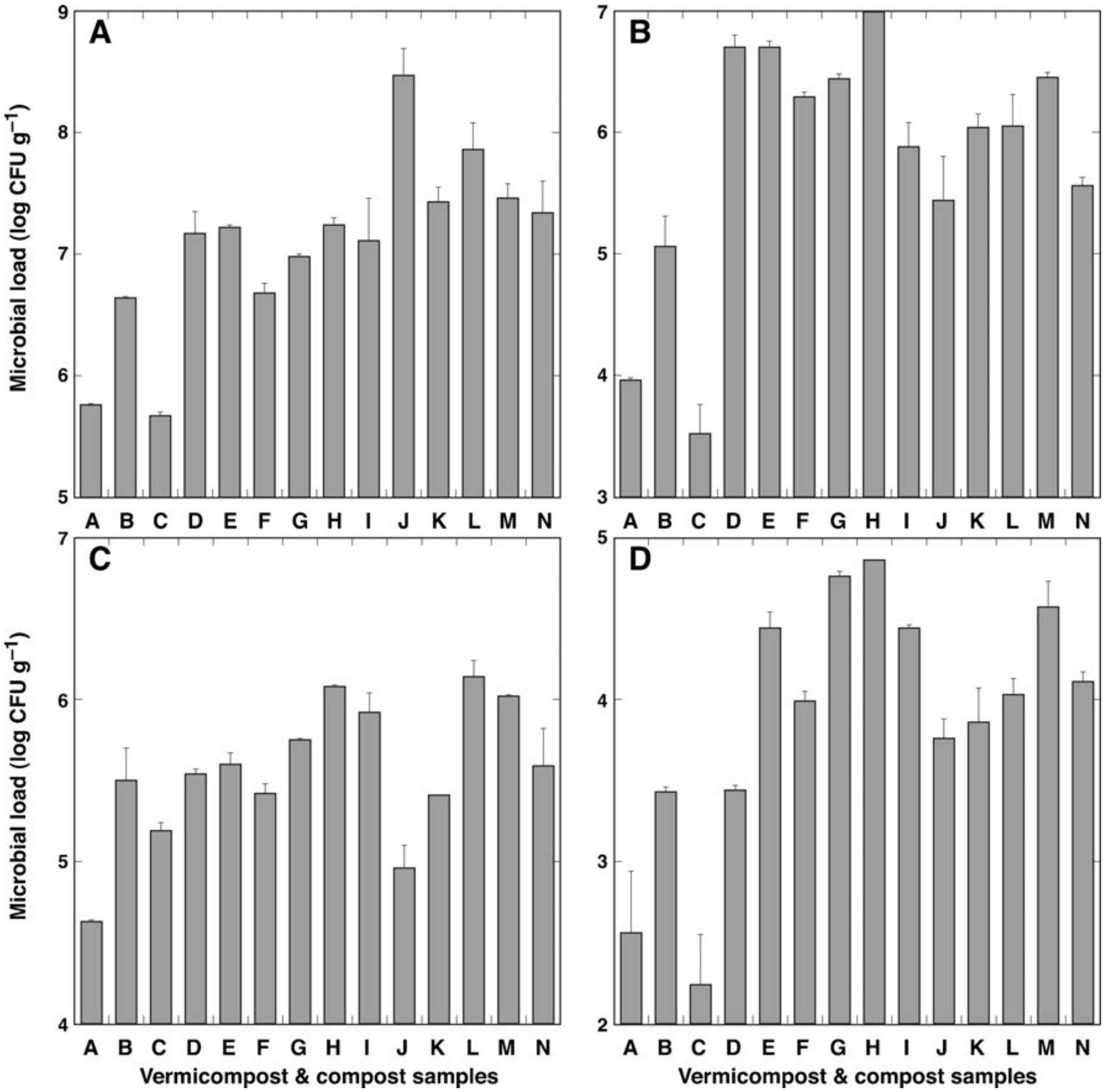

Fig. 2 Microbial variables of vermicompost and compost samples. **a** total number of bacteria; **b** total number of coliforms; **c** total number of cultivable filamentous fungi; **d** number of potentially pathogenic fungi. Data are expressed on dry mass basis as means from two independent measurements ± SD. Abbreviations of sample names as in Table 1

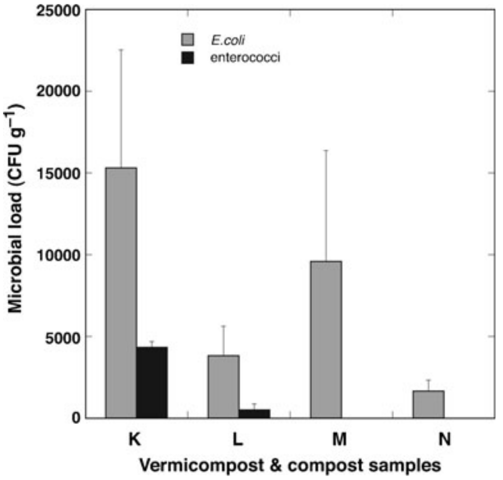

Fig. 3 Number of *E. coli* and enterococci in composted sewage sludge (*K*) and vermicompost samples made from it at different maturity levels (*L* to *N*). Data are expressed on dry mass basis as means from two independent measurements ± SD. Abbreviations of sample names as in Table 1

Providencia rettgeri (sewage sludge, sample K); *Escherichia hermanii* (L), and *Citrobacter koseri* (N).

Table 3 summarizes the prevalence of dominant fungal genera isolated on RBA and Mycosel agar. Six samples (B, D, H, I, M, and N) contained the potentially pathogenic fungal species *Aspergillus fumigatus* at the level of 5.62×10^2 to 1.00×10^4 CFU g^{-1} DM. rRNA gene sequencing showed that six samples (B, D, E, F, K, L) contained *Pseudallescheria boidii*. Also, *Pseudallescheria fimeti* was isolated from two samples (B, D), and sample B contained *Pseudallescheria minutispora*. Samples G and M contained *Scedosporium apiospermum*, and samples G and D contained *Scedosporium prolificans*. Sample F contained *Scopulariopsis brevicaulis*. Sample L contained *Stachybotrys chartarum*. Samples A and J contained *Arthrographis kalrae*. Other potentially pathogenic fungal species detected were *Geotrichum* spp. (A, I, M) and keratinolytic species *Aphanoascus terreus* (B) and *Doratomyces columnaris* (M, N).

Comparison of the levels of potentially pathogenic fungi in compost feedstocks (D and K) with those in final products (E, F, N) suggests that earthworm activities did not decrease the levels of potentially pathogenic fungi. In contrast, the levels were higher in mature vermicomposts than in the respective substrates.

Regarding plant growth-promoting fungi, members of the genus *Mortierella* (*Mortierella alpina*, *Mortierella polycephala*) that produce arachidonic acid, a suppressive agent of plant pathogenic fungi, were isolated only from samples of two manufacturers (first and third). Members of the *Trichoderma* genus (*Trichoderma atroviride*, *Trichoderma citrinoviride*), which are antagonists of plant pathogenic fungi, were isolated from several samples, e.g., C, F, G, H, and J to N. *Trichoderma* spp., and members of the genera *Aspergillus* (*Aspergillus candidus*, *Aspergillus fumigatus*, *Aspergillus niveoglaucus*, *Aspergillus repens*, *Aspergillus sclerotiorum*, *Aspergillus versicolor*), *Mucor* (*Mucor circinelloides*, *Mucor racemosus*), *Paecilomyces*, *Penicillium* (*Penicillium commune*, *Penicillium concentricum*, *Penicillium ilerdanum*), and *Verticillum* (*Verticillum luteo-album*) were isolated from six to 12 of analyzed samples.

Factors affecting microbial abundance

There was a relatively high correlation between moisture content and the total number of coliforms, the total number of cultivable filamentous fungi, and the number of potentially pathogenic fungi (r=0.76, 0.74, and 0.70, and R^2=0.58, 0.55, and 0.48, respectively). The correlation between the moisture content and the total number of bacteria was weak (r=0.27).

In samples A to J, pH was positively correlated with total number of coliforms (r=0.89, R^2=0.80). In samples K to N, this correlation was weak (r=0.45, R^2=0.20). In addition, in samples A to J, there was medium strong correlation also between pH and other microbial variables of the vermicompost, r reaching 0.71 (R^2=0.50), 0.64 (R^2=0.41), and 0.76 (R^2=0.58) for the total number of bacteria; the total number of cultivable filamentous fungi; and the number of potentially pathogenic fungi, respectively. In samples K to N, correlation between pH and microbial variables differed: r=0.80, R^2=0.64 for the total number of bacteria; r=0.99, R^2=0.99 for the total number of cultivable filamentous fungi; and r=0.45, R^2=0.20 for the number of potentially pathogenic fungi.

Evidently, the nature of feedstock used significantly affected the amounts of cultivable microorganisms of several groups. Thus, the number of potential pathogenic fungi was the highest in samples G and H (Fig. 2d, p<0.0001), where abscised tree leaves were used for feeding, in spite of different additions (organic waste vs. cow manure). These vermicompost samples also had the highest number of total filamentous fungi (Fig. 2c, p=0.03). Vermicompost sample J, which was produced from starchless potato pulp and composted grass, had the highest number of total bacteria.

Discussion

Biological activity of compost and vermicompost extracts

Besides nutritional and other beneficial effects on plants, it is recognized that vermicompost can affect plant growth through the high content of biologically active substances produced by a complex activity of earthworms and microorganisms (Krishnamoorthy and Vajranabhaiah 1986; Tomati et al. 1988). In addition, a potential plant growth-affecting activity of composts has been associated with plant growth-promoting

Table 3 The dominant fungal genera isolated on Rose Bengal and Mycosel agars and estimated according to the morphological characteristics, and their levels in analyzed samples, from A to N (log CFU g^{-1} dry mass)

Genus	A	B	C	D	E	F	G	H	I	J	K	L	M	N
On Rose Bengal agar														
Acremonium	–	–	–	–	–	–	–	–	–	4.11	–	–	–	5.42
Aspergillus	2.03	3.19	4.93	–	5.10	–	–	5.35	5.07	4.85	–	–	–	4.81
Bionectria	–	–	–	–	–	–	–	–	–	–	–	5.00	–	–
Botrytis	–	–	–	–	–	–	–	–	–	2.11	–	–	–	–
Cladosporium	–	–	–	–	–	–	–	–	–	4.11	–	–	–	–
Doratomyces	–	–	–	–	–	–	–	–	–	4.89	–	–	4.99	5.01
Fusarium	–	–	–	–	–	–	–	–	–	3.00	–	5.00	–	–
Geomyces	–	5.15	–	–	–	–	–	–	–	4.11	–	–	–	4.00
Geotrichum	4.33	–	–	–	–	–	–	–	–	–	–	–	–	–
Metarhizium	–	–	–	–	–	–	–	–	5.03	–	–	–	–	–
Mortierella	2.03	5.15	2.03	–	–	–	–	–	–	4.00	–	–	–	–
Mucor	–	–	–	3.20	5.10	5.02	3.18	5.09	4.33	2.11	4.75	3.45	3.42	4.16
Mycogone	–	–	–	–	–	–	–	–	–	3.11	–	–	–	–
Paecilomyces	2.03	2.45	–	–	–	4.02	5.50	–	–	–	–	–	–	–
Penicillium	2.77	3.45	4.03	3.20	5.10	5.02	–	5.75	4.07	–	4.75	5.47	4.42	–
Scedosporium	–	–	–	5.00	–	–	5.40	–	–	–	–	–	–	–
Scopulariopsis	–	–	–	5.16	–	–	–	–	–	–	–	–	–	5.42
Sporothrix	–	–	–	–	–	–	5.40	–	–	–	–	–	–	–
Sordaria	–	–	2.00	–	–	–	–	–	–	–	–	–	–	–
Stachybotrys	–	–	–	–	–	–	–	–	–	–	–	5.00	–	–
Trichoderma	–	–	2.03	–	–	5.32	4.44	5.65	–	3.19	3.75	5.05	4.72	4.42
Verticillium	–	–	–	5.33	5.14	5.02	5.92	5.05	–	–	–	–	–	–
White sterile mycelium	5.13	5.15	–	–	–	–	5.62	5.05	5.07	4.28	–	–	–	–
On Mycosel agar											–	–	–	–
Acremonium	–	–	–	–	–	4.70	–	3.44	–	3.51	–	–	–	3.51
Aphanoascus	–	3.05	–	–	–	–	–	–	–	–	–	–	–	–
Arthrographis	1.73	–	–	–	–	–	–	–	–	3.00	–	–	–	–
Aspergillus	–	–	–	3.58	2.46	–	–	5.44	5.35	3.75	3.42	4.42	4.00	–
Aureobasidium	–	–	–	–	–	–	2.32	–	–	–	–	–	–	–
Geotrichum	2.57	–	–	–	–	–	–	–	3.33	–	–	–	3.33	–
Microascus	–	3.15	–	–	–	–	–	–	–	–	–	–	–	–
Paecilomyces	–	–	–	–	–	–	–	–	2.33	–	–	–	2.33	–
Penicillium	–	–	–	–	–	–	–	–	–	–	–	–	3.94	–
Pseudallescheria	–	3.15	–	5.00	3.46	4.40	3.32	–	–	–	3.00	3.00	–	–
Scedosporium							3.00						3.00	
Scopulariopsis	–	–	–	3.39	–	4.00	–	–	–	–	–	–	–	–
Thermomyces	3.00	2.75	2.03	–	–	5.10	–	–	–	–	–	–	–	–
Verticillium	–	–	–	–	–	–	–	4.04	3.35	3.75	4.02	–	–	3.11
Number of genera	8	10	6	7	5	9	9	7	8	14	6	8	8	8

rhizobacteria (Alvarez et al. 1995). Production of hormone-like substances by these bacteria is one of possible beneficial mechanisms of composts on plants. Compost preparations also can be inhibitory to growth and nutrient uptake of plants (Gamliel and Stapleton 1993; Alvarez et al. 1995), e.g., plant growth-inhibiting activity has been attributed to the high level of indole-3-acetic acid produced by rhizobacteria present in some compost samples (Alvarez et al. 1995).

Plant response tests with a compost-amended substrate are often used for evaluation of composts. According to Annex D of the BSI PAS 100 (British Standards Institution 2011), reduction of germination and plant mass above surface of tomato grown in compost should not exceed 20 % when compared with a peat control. The same tests and limits are usually applied to vermicompost testing. However, differences in plant growth can result because of different amounts of mineral nutrients in the respective mixes. In order to avoid complex interactions and possible side effects, soilless test with seedlings germinated from seeds is preferable, using several plant species to account for both stimulating and inhibiting effects of the particular sample. In the present study, by using vegetable crop species with different sensitivities to vermicompost extracts at the seedling stage (Ievinsh 2011), it was possible to thoroughly characterize commercial vermicompost preparations made by various manufacturers with different feedstock and earthworm populations.

Differences in plant growth-affecting activity of different samples due to the extraction procedure were possibly affected by physical characteristics of the particular vermicompost formulation. This could be especially important for vermicompost samples with different relative moisture contents. It is evident that dry-stored vermicompost samples (A and C) were characterized by a lower extractability of putative stimulating substances, together with a higher extractability of inhibitory substances, in spite of identical (equilibrated) moisture contents of samples used for extraction.

In comparison to vermicompost samples, also composted cow manure extract exhibited relatively high plant growth-affecting activity. In general, both composted cow manure and its vermicompost had low activity in respect to hypocotyl growth. However, in the tests with Swedish turnip and carrot seedlings, 10 % cow manure extract showed significantly higher stimulating effects on radicle growth in comparison to the respective vermicompost samples (Table 2).

Microbiological quality of the vermicompost and compost samples

Indicators of fecal contamination, bacteria *E. coli* and enterococci, were detected in sewage sludge (sample K) and in two consecutive immature vermicompost samples (samples L and M; Fig. 3). *E. coli* was present also in mature vermicompost (sample N) at a level of $(1.66 \pm 0.67) \times 10^3$ CFU g^{-1} DM, which is not below 1,000 CFU g^{-1} of DM as defined in the Regulation of the Cabinet of Ministers of Latvia Nr. 530 (2006) for organic composts. This level in mature vermicompost was 89.2 % lower than in sewage sludge. As enterococci should not exceed 1,000 CFU g^{-1} DM and *Salmonella* spp. should be absent in 25 g of the sample, consequently, all analyzed vermicompost samples, with the exception of that produced from sewage sludge, met these qualifications. According to USEPA (1995) guidelines, the fecal coliform limit for biosolid of excellent quality must be <1,000 CFU g^{-1} and the limit for *Salmonella* spp. is <3 CFU g^{-1} (Contreras-Ramos et al. 2005). In the Specification for Composted Materials in the UK (British Standards Institution 2011), it is defined that the CFU number of *E. coli* should not exceed 1,000 CFU g^{-1}, when determined at 44 °C incubation temperature, and *Salmonella* spp. must be absent in 25 g of sample.

Several enterobacteria were isolated from the samples: *Providencia rettgeri* (sewage sludge, sample K), *E. hermanii* (L), and *Citrobacter koseri* (N). Members from the genus *Citrobacter* were isolated from animal manure and compost as mesophilic and thermophilic organisms (Ryckeboer et al. 2003). *Citrobacter* spp. and *Providencia rettgeri* have been reported to be resistant to heavy metals (Macaskie et al. 1994; Hassen et al. 1998), which, in our case, were present in sewage sludge in elevated concentrations (1,418 mg kg^{-1} Zn, 226 mg kg^{-1} Cu, 42 mg kg^{-1} Pb, 2.2 mg kg^{-1} Cd, 25 mg kg^{-1} Ni, 5 mg kg^{-1} Hg; Ievinsh et al., unpublished data).

Since there are no standards for the level of total coliforms, data from other studies can be used for comparison. In the investigation of Monroy et al. (2008) in Spain where pig slurry was composted using four different earthworm species, the number of total coliforms in the pig slurry was 7.23×10^5 CFU g^{-1}, which in comparison was significantly lower than found in the composted cow manure (sample D) and after vermicomposting decreased to 1.00×10^5 CFU g^{-1} in the case of *Lumbricus rubellus* and even lower in the case of other earthworm species.

Composted cow manure (sample D) contained 5.03×10^6 CFU g^{-1} DM of total coliforms, and after vermicomposting (sample E), the level did not change. Only in the final product, the sieved fraction of the vermicompost (F), a significant reduction (by 61.4 %, $p=0.005$) of the coliform level (1.94×10^6 CFU g^{-1} DM), was observed. A low level of coliforms (under 1.00×10^5 CFU g^{-1}) was detected only in the dry vermicompost samples A and C, which were also produced from cow manure, but by a different manufacturer. In the case of sewage sludge, significant reduction of total coliforms (by 67.5 %) was detected only in mature vermicompost (Fig. 2b).

There are also no standards for the level of other bacteria and fungi. In the investigation of Gopal et al. (2009) in India, the level of aerobic heterotrophic bacteria counted on nutrient agar in vermicompost samples produced from cow manure was 10×10^6 CFU g^{-1} of fresh sample. From our samples, vermicompost A and B had a remarkably lower level of total number of bacteria, and vermicompost J had a remarkably higher level, in comparison to the study of Gopal et al. (2009), which was even higher than the level of bacteria in sewage sludge (sample K). It appears that in order to reduce the level of undesirable microorganisms in vermicompost, it should be stored dry, as microbial propagules other than spores are no longer present in air-dried vermicompost samples. This is supported by the study of Fracchia et al. (2006) on bacterial populations in vermicompost during long-term storage, where they remained unchanged even in vermicompost sample stored for 12 years with the moisture content from 2.89 to 6.00 %.

In the study of Anastasi et al. (2005) in Italy where fungal populations of the vermicompost produced from 70 % dung (from cows, poultry, and various zoo animals) and 30 % plant debris from various sources were investigated, it was estimated that number of fungi was up to $4.0\times10^{5}\,\mathrm{CFU\,g^{-1}}$. Samples A, C, and J had fungal CFU numbers below that level, but others were close to it or even exceeded this level.

Several samples contained potentially pathogenic fungal species, such as *Aspergillus fumigatus*, *Pseudallescheria* spp., and *Scedosporium* spp. Fungi from genus *Pseudallescheria* with their anamorphs *Scedosporium* have been described as human pathogens (Rainer et al. 2000; Lu et al. 2011). *Scopulariopsis brevicaulis* is reported as a multiresistant human pathogen (Cuenca-Estrella et al. 2006). *Stachybotrys chartarum* is known as a producer of various toxins (Kuhn and Ghannoum 2003). Samples A and J contained *Arthrographis kalrae*, which can be pathogenic for humans (Perlman and Binns 1997; Chin-Hong et al. 2001). Other potentially pathogenic fungal species detected in several samples were *Geotrichum* spp. (Cooke and Kabler 1955) and the keratinolytic species *Aphanoascus terreus* (Sugiyama and Mikawa 2001) and *Doratomyces columnaris* (Maghraby et al. 2008). All of these mentioned species have been isolated from vermicomposts and composts previously (Ryckeboer et al. 2003; Anastasi et al. 2005).

When comparing the level of potentially pathogenic fungi of compost feedstocks (D and K) with final products (E, F, N), it can be seen that earthworm activities did not decrease the level of potentially pathogenic fungi, as they were higher in mature vermicomposts than in the respective substrates. A similar problem has been described also in other studies, e.g., in composts produced from biowaste, pulp trash, mushroom farm waste, and poultry and pig manure (Beffa et al. 1998). The abundance and frequency of occurrence of particular microfungi of medicinal interest from genera *Aspergillus* (*Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus flavus*), *Fusarium* (*Fusarium oxysporum*, *Fusarium moniliforme*), and *Chrysosporium* spp. were higher in vermicompost than in compost without earthworms.

Members of the genus *Mortierella* (*Mortierella alpina*, *Mortierella polycephala*), which produce arachidonic acid, a suppressive agent of plant pathogenic fungi (Eroshin and Dedyukhina 2002; Fakas et al. 2009), were isolated only from samples of two manufacturers (first and third). Members of the *Trichoderma* genus (*Trichoderma atroviride*, *Trichoderma citrinoviride*), which are antagonists of plant pathogenic fungi, were isolated from several samples, e.g., C, F, G, H, and J to N. Members from the genera *Aspergillus*, *Mucor*, *Paecilomyces*, *Penicillium*, *Trichoderma*, and *Verticillum* were isolated from six to 12 of analyzed samples, each in general reflecting the broad spectrum of degradable compounds by these fungi (Ryckeboer et al. 2003). The presence of several genera (*Aspergillus*, *Cladosporium*, *Mucor*, *Penicillium*, *Trichoderma*, and *Verticillium*) in vermicompost samples I and J produced from potato pulp is known to be degraders of starch (Ryckeboer et al. 2003) that could remain in the feedstock used for vermicomposting (Mayer and Hillebrandt 1997).

Relationship between microbial diversity and plant growth-affecting activity

Results of stepwise multiple regression analysis show that of all the variables included in the model, only the total number of cultivable filamentous fungi and the number of fungal genera in vermicompost and compost samples had significant positive impact on the summed stimulating activity of the extracts on plants (Table 4). The total number of coliforms had negative impact on the stimulating activities and positive impact on inhibiting activities. This can be explained by the fact that the total number of coliforms might be an indicator of the maturity of the vermicompost (Yadav et al. 2010). Total number of bacteria had significant negative impact, and moisture content of samples had positive impact on summed inhibiting activity. Considering fungal genera that were present in at least four samples, the number of CFU from the genera *Aspergillus* on Mycosel agar and *Verticillium* on Rose Bengal agar with chloramphenicol (RBA) had medium strong or weak positive correlation with stimulating activity, $r=0.61$ and 0.31, respectively. Other dominant fungal genera had negative correlation with the stimulating activity with the r values −0.56 (*Pseudallescheria*), −0.53 (*Mucor*), −0.51 (*Verticillium* on Mycosel), −0.42 (*Trichoderma*), −0.36 (*Penicillium*), and −0.29 (*Paecilomyces*). A stimulating effect of the genus *Aspergillus* member *Aspergillus niger* on the growth rate of clover *Trifolium repens* when added to sugar beet waste supplemented with rock phosphate has been shown (Vassilev et al. 1996).

It can be argued, however, that in the present experiments, microbial diversity was analyzed in the vermicomposts but vermicompost extracts were used for measurement of plant growth-affecting activity. However, based on molecular analysis, it was found that microbial diversity of vermicompost samples was strongly associated with that in vermicompost extracts produced from them (Fritz et al. 2012). Any water-soluble products of microbial activity from vermicompost samples eventually will be present in the respective extracts. The multiple R^2 values for summed stimulating and inhibiting activity (0.74 and 0.89, respectively) do show that there are other factors affecting plant growth, which were not tested in this investigation. However, the described tests for plant growth-affecting activity using water extracts reflect the overall activity of vermicompost samples and can be used for

Table 4 Results of stepwise multiple regression analysis of microbiological variables and plant growth-affecting activity of vermicompost samples

Variable name	Coefficient±SE	*t* value	*p* value
Summed stimulative activity			
Total number of coliforms (CFU g^{-1} DM)	−174.62±50.51	−3.46	0.006
Total number of bacteria (CFU g^{-1} DM)	Not significant		
Total number of cultivable filamentous fungi (CFU g^{-1} DM)	343.23±123.79	2.77	0.02
Number of potentially pathogenic fungi (CFU g^{-1} DM)	Not significant		
Number of fungal genera	78.01±17.88	4.36	0.001
Moisture content (%)	Not significant		
pH of extract	Not significant		
	p value of the final model is 0.003, multiple R^2 is 0.74		
Summed inhibitory activity			
Total number of coliforms (CFU g^{-1} DM)	152.72±21.75	7.02	<0.0001
Total number of bacteria (CFU g^{-1} DM)	−120.93±20.76	−5.83	<0.0003
Total number of cultivable filamentous fungi (CFU g^{-1} DM)	−93.06±40.97	−2.27	0.05
Number of potentially pathogenic fungi (CFU g^{-1} DM)	Not significant		
Number of fungal genera	Not significant		
Moisture content (%)	−3.89±0.87	−4.49	0.002
pH of extract	Not significant		
	p value of the final model is 0.0003, multiple R^2 is 0.89		

testing of biological activity of waste-derived compost and vermicompost preparations.

Cluster analysis was performed to identify similarities of microbial and plant growth-affecting activity of vermicompost samples and substrates. The cluster analysis revealed grouping of vermicompost samples according to the manufacturer and substrate used (Fig. 4). Substrate and vermicompost samples from the second manufacturer (D, E, F) formed a distinctive group differing from the rest of samples. A second distinctive group was formed from sewage sludge and immature and mature vermicompost samples made from the sludge (K, L, M, N). A third distinctive cluster was formed by stored dry vermicompost samples of the first manufacturer.

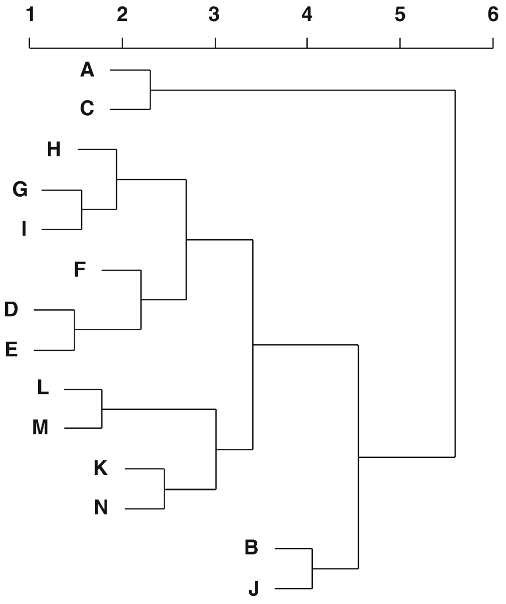

Fig. 4 The cluster analysis of vermicompost and compost samples using maximal distance. Abbreviations of sample names as in Table 1

Significant manufacturer-associated differences in biological activity and microbial composition of vermicompost samples most likely are due to the particular type of earthworm population used for vermicomposting and to conditions of vermicomposting. In addition, low microbial content in air-dry samples most probably was due to the fact that microbial propagules (mycelial fragments, living bacteria, etc.) other than spores were no longer present in these samples. It could be suggested that vermicompost should be stored dry, in order to reduce the level of undesirable microorganisms.

In conclusion, the results of the present study clearly indicate that vermicompost screening can reveal significant differences in biological properties between analyzed samples regarding effects on seedling growth and presence of potentially pathogenic bacteria and filamentous fungi. Analysis of this type can be useful for functional studies aiming to determine factors affecting quality characteristics of vermicompost preparations in a sequence feedstock > processing > final product. The relationships between plant growth-affecting substances in vermicompost samples and activity of particular groups of microorganisms need to be identified. The described system can be used for testing of microbiological quality and biological activity of organic waste-derived composts and vermicomposts.

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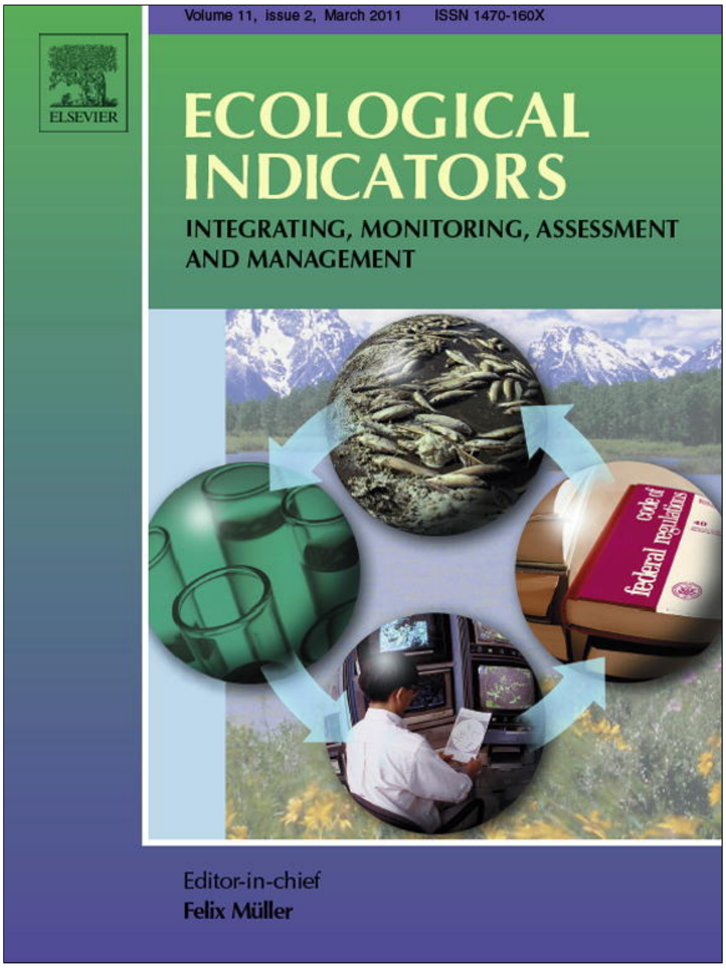
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Linking woodland key habitat inventory and forest inventory data to prioritize districts needing conservation efforts

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ABSTRACT

Woodland key habitat (WKH) inventories have been conducted in northern European countries, with the aim to create networks of minimally disturbed forest stands for protection. The goal of national forest inventory is to provide information relevant to forest management, such as on forest types, trees species composition, age structure and wood volume. The aim of this study was to link these two inventory databases to identify districts of Latvia most deficient in connectivity and habitat quality, in order to prioritize districts needing conservation effort. As an example, the area of deciduous forest with nemoral tree species (oak, ash, lime, maple and elm) and aspen was chosen. These forests provide habitat for a specific community of epiphytes. Using information in the WKH database, habitat quality in different districts of Latvia was estimated by the frequencies of occurrence of structural elements and selected indicator epiphyte species in nemoral tree species and aspen WKHs. Using digital data in the national forest inventory database, fragmentation metrics were determined for forests that, according to age and tree species composition, could potentially be nemoral tree and aspen WKHs. On a regional level, the lowest habitat quality in WKH occurred in districts that had the least fragmentation of potential WKH forest. In the less fragmented areas, the habitat quality of the existing WKH will likely increase in the future, and could be promoted by management to create structural elements typical of natural forests. The districts with the most fragmented nemoral and aspen forests, contained WKHs with the best habitat quality. A focus on protection should be given to these stands as they are the most likely to support source populations, and there is a need to improve spatial continuity of suitable tree substrate in these areas.

1. Introduction

Conversion of land-use for agricultural production has led to large-scale fragmentation of forest ecosystems. Old-growth forests (sensu Wirth et al., 2009) have been almost completely lost in hemiboreal Europe, causing loss of species. For example, it has been estimated that 73% of the known forest species in Finland have become extinct, and further loss due to past human disturbances is expected (Hanski, 2000). Habitat fragmentation decreases the total habitat area available to species, and also decreases connectivity, reduces patch size, and increases edge effects (Laurence, 2008). There is a need to identify connectivity and landscape structure features required for conservation of the species requiring old growth forest habitat (Sutherland et al., 2006). Parallel to habitat loss, intensive forestry has caused changes in forest structure at different spatial levels (Kuuluvainen, 2002). In particular, there has been a decrease in density of large, old trees, and in the amounts of standing and fallen dead wood (Siitonen et al., 2000).

However, operational targets at landscape levels for species, structures and processes that are typical of natural forests are generally lacking, which is partly due to insufficient knowledge (Branquart et al., 2008). It is recognized that conservation of biodiversity requires knowledge of the minimal amounts of habitat needed for a particular species (Fahrig, 2001; Angelstam et al., 2004a). Knowledge on habitat thresholds regarding size, habitat quality and spatial connectivity needs to be transformed into performance targets at different levels (e.g. patch size and spatial arrangement) and landscape scales, which can differ depending on the amount of non-degraded habitat, target species and level of ambition of forest managers (Angelstam et al., 2004b).

Old-growth nemoral deciduous (pedunculate oak *Quercus robur*, European ash *Fraxinus excelsior*, lime *Tilia cordata*, Norway maple *Acer platanoides*, Wych elm *Ulmus glabra* and European white elm *Ulmus laevis*) forest has been almost completely converted to intensive agricultural land-use in Europe, and the most natural forest of this type may be found in the Bialowieza Reserve in Poland

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(Peterken, 1996). In Latvia, some patches of near-natural nemoral deciduous forest remain on Moricsala Island (Brūmelis et al., 2011), but this habitat has been severely fragmented, and presumably all the existing stands have been cut at some time and/or used for agricultural purpose. For example, the area of oak forest is only 0.8% of the total forest area in Latvia (State Forest Service, 2007). The richness of red-listed species in nemoral deciduous forests has been shown to be decreased in landscapes where the area of this habitat is smaller (Paltto et al., 2006).

One of the conservation efforts to maintain biodiversity of forests has been the formation of woodland key habitat (WKH) networks, which are aimed to protect core areas with high biodiversity (Perhans et al., 2007). In the southern boreal zone of Europe, most WKH show sign of exploitation during the past 150 years, leading to lower amounts of important structural elements, such as large diameter coarse wood debris (Ericsson et al., 2005; Jönsson et al., 2009). A key group of organisms for identification of WKH are epiphytes, particularly in nemoral deciduous forests. Nemoral tree species with nutrient rich bark and also aspen (*Populus tremula*) are associated with particularly high richness of epiphytic bryophytes and lichens (Löbel et al., 2006a; Lõhmus et al., 2007; Mežaka et al., 2008). Species richness of lichens is also related to the amount of old deciduous trees in a stand (Uliczka and Angelstam, 2000). Loss of habitat connectivity due to forest clearcutting limits occupancy of many epiphytic species at different scales (Baldwin and Bradfield, 2007; Öckinger et al., 2005; Snäll et al., 2004), depending on their mode of dispersal (Löbel et al., 2006b; Löbel and Rydin, 2009). Lichen epiphytes typical of late successional forest are sensitive to a break in forest temporal continuity, due to unsuitable substrate quality, lack of preferred tree species, and a long period of time required for colonization (Fritz et al., 2008). Less-intensive harvest that involves retention of green-trees can provide refugia for some of the species tolerant to increased exposure to sunlight, thereby acting as stepping stones for colonization (Hedenås and Hedström, 2007).

In Latvia, the WKH database includes information on indicator species recorded in the stands, and on the presence/absence of structural elements indicative of minimal human disturbance. The State Forest Register contains stand-level information used in forest management, which is digitized as GIS layers. Thus, information in the two databases can potentially be used to derive indicators for districts based on differences in the frequency of occurrence of indicator species, in relation to habitat quality and connectivity. The present study aims at using information from these databases on nemoral deciduous tree and aspen stands to (1) determine the stand-level relationship between the presence of WKH indicator species and occurrence of structural elements, (2) identify differences between districts in connectivity and habitat quality, and (3) determine if the differences in frequency of occurrence of indicator species among districts can be explained by connectivity and habitat quality. This was done to rank districts based on landscape-and stand-scale deficiencies in quality, and to thereby make recommendations for conservation efforts on a district basis.

2. Materials and methods

2.1. Site description

The climate in Latvia is characterized by rather mild winters (mean January temperature −3 to −4 °C), cool summers (mean July temperature 16.5 °C) and a vegetation period beginning in mid-April, lasting 180–200 days (Kļaviņš et al., 2008).

Latvia is located in the boreo-nemoral region, where the dominant coniferous forests are interspersed by nemoral deciduous tree woodland (Sjörs, 1963). The most common nemoral deciduous trees in Latvia, in order by decreasing forest area, are: *Fraxinus excelsior*, *Quercus robur*, *Tilia cordata*, *Acer platanoides*, *Ulmus glabra* and *Ulmus laevis*. The pioneer trees aspen *Populus tremula* and birch *Betula* sp. are abundant, together contributing 22% of the timber volume (State Forest Service, 2007).

In Latvia, clear-cutting has been the main method used to cut timber in forests that have reached cutting age. Clear-cut size has been less than 5 ha, or sometimes larger in cases when seed trees are used for regeneration of pine.

We lack data on the past land-use in the districts, but some of the stands probably were utilized previously as wooded meadows and pasture. This type of land-use was rapidly abandoned during the 1940s with the onset of farm collectivization. In Latvia, it is considered that at about 70 years after the end of use of a forest for agriculture, the area has lost the biological value of open woodland, and has gained value as a closed forest with the respective shade-tolerant species (Larmanis, 2010). There are no plans for widespread rejuvenation of traditional wooded pasture practices in Latvia, except in a few areas for which such projects have been planned. Therefore, the conservation value of these forests in Latvia needs to be viewed from a perspective of closed canopy forest, and not traditional wooded meadows and pasture.

2.2. Woodland key habitat inventory data

In Latvia, the criteria for designation of a WKH are based on the presence of species (e.g. bryophytes, lichens and insects) considered to require spatial and/or temporal continuity of natural forest processes, and also on the amounts of structural elements that indicate minimal human disturbance (Auziņš, 2001). The WKH inventory, which began in 1997, was carried out by about 170 persons, each of whom was assigned a specific area. Training and calibration courses were organized for the persons involved. The WKH database contains data collected in the field on structural elements (e.g. dead wood, tree hollows and fire scars) and indicator species. The WKHs are cross-referenced with forest inventory data on the respective stands (see Section 2.3 below). As not all forests meeting WKH criteria have yet been identified (Anon., 2005), the inventory process is ongoing.

Stands designated as nemoral woodland key habitats were extracted from the WKH database. We additionally included also stands classified as another WKH type, but in which a nemoral tree species occurred in the overstorey. Since many epiphytes of nemoral tree species also utilize aspen as a substrate (Mežaka et al., 2008), stands with this species were also considered. WKHs with aspen contributing ≥70% of the wood volume (data from the forest inventory register) were compiled in a separate data set together with the nemoral tree WKH.

A total of 53 species (polypores, mosses and liverworts, lichens, vascular plants, beetles and snails) have been used in practice in the WKH inventory as indicator species, although the complete list includes 212 species. The abundance of indicator species in WKHs is estimated on a 3-point scale. The aim of the inventory was to survey a large area in a short period of time to identify sites for protection. Therefore, a thorough check for all species on the indicator list was not made in each stand. In cases when, for example, four indicator species had been found, and forest structure was indicative of natural processes, more intensive checks for more species were usually not made. Therefore, we restricted the list of species in this study to epiphytic bryophytes and lichens that are typically found on nemoral tree species and aspen bark, and which could be easily found and identified in the field by the inventory workers (Table 1). Species (e.g. *Graphis scripta*) abundant in all districts in the survey were also excluded, as they were considered to be insensitive to habitat quality and fragmentation. In total 4 lichen and 7

Table 1
Indicator species and structural elements of woodland key habitats used in the study.

Species	Structural elements
Lichens	Uneven age structure
Acrocordia gemmata	Abundant downed wood in many decay stages
Arthonia leucopellea	Abundant dead standing trees
Arthonia spadicea	Abundant polypores
Lobaria pulmonaria	At least four nemoral tree species
Bryophytes	Trees with hollows
Anomodon spp.	
Homalia trichomanoides	
Jamesoniella autumnalis	
Lejeunea cavifolia	
Metzgeria furcata	
Neckera complanata	
Neckera pennata	

bryophyte species (*Anomodon* species were grouped as the genera in the inventory) were used in the study.

Information on forest structure was recorded in each WKH for 25 structural elements on a presence/absence scale. From this list, we included six (uneven age structure, abundant standing and downed dead wood, abundant polypores, at least four nemoral tree species, trees with hollows) in the study (Table 1). The criteria used for selecting these six structural elements were: (1) presence in a part of the selected stands, (2) relevance to nemoral and aspen forests, (3) easy to estimate and calibrate across persons conducting inventory and (4) lack of covariance with other elements. The excluded elements were absent or rare in the selected nemoral and aspen forests (e.g. beaver activity), specific for other WKH types (e.g. presence of springs, black alder on raised hummocks and fire scars), they were clearly covariate with other variables (e.g. abundant dead wood in a few versus many decay stages—one or the other was chosen), or observed to be difficult to calibrate between workers (e.g. canopy openings).

The frequencies of occurrence of the species and the six selected structural elements (Table 1) in WKHs were calculated by administrative district. District size ranged from 163 000 ha in Bauska to 359 000 ha in Liepāja (Table 2). Division by administrative district (Fig. 1) was chosen because the State Forest Register and woodland key habitat inventory databases were organized in this way.

Two districts (Kraslava and Riga) were at onset rejected from the study. Inventory had practically not been conducted in the former due to lack of local qualified personnel. The latter district contains a large National park, in which inventory data were not integrated in the WKH database.

2.3. Forest inventory data acquisition and processing

The State Forest Service maintains a register of stand-level inventory data on tree species composition, age, density, relative timber volume, and size. We use the term stand to define the smallest management unit in the forest register, which is a forest area relatively homogenous in tree species composition, size and age. Stands size ranges from a minimum of 0.1 ha to over 40 ha, but 80% of stands are under 2 ha in size. The textual information in the register is linked with digital map information as GIS layers. The wood volume in a stand is given on a relative scale in 10% classes for each species within tree layers. Age is also given for each tree species by canopy layers. The overstorey species are ranked in importance by volume and density. To determine fragmentation patterns of stands that could potentially be nemoral tree and aspen WKHs, we extracted two sets of data from the State Forest Service Register using ArcGIS software:

1. all stands with a nemoral tree species (oak, lime, ash, maple and elm) ranked as one of the three most important species in the overstorey and with age ≥100 years;
2. all stands with a nemoral tree species (oak, lime, ash, maple and elm) ranked as one of the three most important species in the overstorey and age ≥100 years plus all aspen stands with a relative volume ≥70% and age ≥60 years.

For each data set, a digital map database was constructed for each of the administrative districts of Latvia. Data were imported

Table 2
Landscape characteristics of the study area. Relative area is given for old nemoral tree forest ≥100 years of age and old nemoral forest ≥100 year plus old aspen (*Populus tremula*) forest ≥60 years of age, as well as area and density of for nemoral tree WKHs and nemoral tree + aspen WKH. Nemoral tree species included are oak *Quercus robur*, ash *Fraxinus excelsior*, lime *Tilia cordata*, maple *Acer platanoides*, Wych elm *Ulmus glabra* and European white elm *Ulmus laevis*.

District	Area (ha)	Forest area (%)	Nemoral forest			Nemoral forest + aspen		
			Area old nemoral years (%)	WKH area (%)	WKH (no. ×10 000 ha^{-1})	Area old nemoral + aspen (%)	WKH area (%)	WKH (no. ×10 000 ha^{-1})
Aizkraukle	256 758	52.5	0.18	0.18	9.5	0.32	0.24	13.4
Alūksne	224 312	55.3	0.12	0.08	5.1	0.37	0.20	11.6
Balvi	237 654	40.4	0.12	0.06	2.5	0.38	0.09	4.4
Bauska	188 344	29.7	0.14	0.05	2.5	0.26	0.07	3.8
Cesis	297 819	51.9	0.31	0.02	1.3	0.57	0.04	2.4
Daugavpils	251 749	34.0	0.16	0.08	3.4	0.35	0.20	9.4
Dobele	163 123	28.6	0.22	0.29	15.3	0.30	0.33	16.7
Gulbene	187 137	50.8	0.20	0.38	19.6	0.35	0.42	22.2
Jekabpils	299 376	41.8	0.05	0.08	4.0	0.18	0.25	12.0
Jelgava	160 282	28.8	0.16	0.40	16.9	0.20	0.41	18.2
Kuldīga	250 329	48.5	0.35	0.10	5.3	0.44	0.12	6.8
Liepaja	359 117	43.1	0.41	0.13	6.8	0.45	0.13	7.4
Limbaži	259 959	49.2	0.31	0.15	9.4	0.44	0.18	11.8
Ludza	240 820	35.6	0.07	0.01	0.7	0.28	0.04	1.9
Madona	334 179	42.2	0.26	0.07	3.5	0.53	0.11	5.8
Ogre	184 239	46.7	0.28	0.10	7.1	0.43	0.13	15.3
Preiļi	203 539	28.0	0.13	0.04	1.4	0.26	0.08	2.8
Rezekne	281 289	30.1	0.09	0.01	0.4	0.29	0.02	1.6
Saldus	217 914	51.0	0.19	0.03	1.9	0.39	0.10	6.0
Talsi	274 727	42.0	0.39	0.07	3.3	0.47	0.10	5.5
Tukums	245 052	55.4	0.24	0.03	2.4	0.33	0.03	0.06
Valka	245 052	38.8	0.25	0.16	13.6	0.36	0.16	0.20
Valmiera	236 120	51.6	0.25	0.04	2.8	0.35	0.04	0.06
Ventspils	245 789	43.3	0.22	0.03	2.5	0.26	0.03	0.04

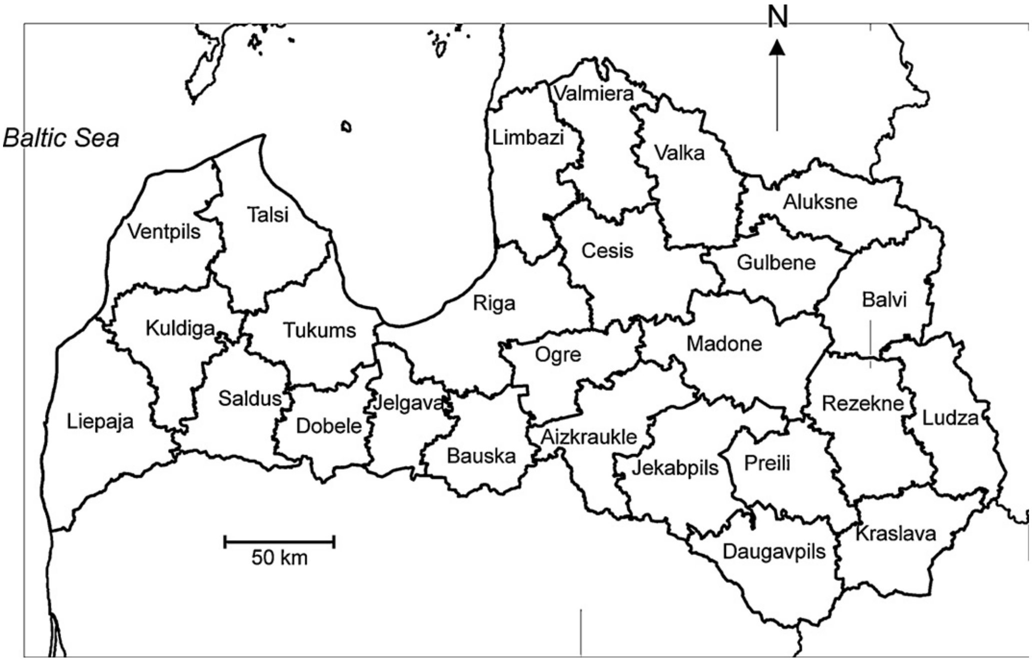

Fig. 1. Administrative districts in Latvia.

from the forest register as ArcGIS image files. A forest digital map for each district was converted to an image file using ArcGIS Conversions Tools—Feature to Raster. The image raster file output resolution was 10 m with one unique raster value id for the selected habitats. The following Fragstats metrics were calculated by district for both of the two filtered databases:

- Total patch area (ha × 10 000 ha^{-1})
- Patch density (number × 10 000 ha^{-1})
- Mean patch area
- Total core area (ha × 10 000 ha^{-1})
- Core area density (number × 10 000 ha^{-1})
- Mean core area of a patch
- Mean Euclidean distance between patches

A patch was defined as an area of the selected forest habitat type surrounded by areas with a different habitat type. Core area was the patch area excluding a 50-m edge around each patch.

2.4. Comparability of data sets

The minimum ages for stands in the data sets were chosen to match the range of age in the greater part of stands in the WKH inventory. About 70% of the total area of stands identified as WKH had an age over 100 years. We considered that setting a lower age threshold would make the data sets much less comparable, i.e. there was much less chance that younger stands would qualify as WKHs. Also, preliminary testing showed that the differences in fragmentation among the districts were similar for data sets using $\geq$100-year and $\geq$80-year thresholds for nemoral forest. Thus, we considered only stands with age $\geq$100 years, while recognizing that younger stands might also qualify as WKHs. The selection of stands with a nemoral species ranked as one of the three most important species in the overstorey encompassed 87% of the total area of stands that had a nemoral species in the overstorey. The relative timber volume of nemoral tree species in the stands selected from the inventory data was in the range of that in the selected WKHs. Therefore, the selected data sets can be considered to be comparable.

2.5. Multivariate data analysis

Indicator species analysis (Dufrêne and Legendre, 1997) was performed to determine if WKH differed in presence and/or abundance of the selected indicator species, depending on presence/absence of each of the structural elements. Indicator values of species were calculated (Dufrêne and Legendre, 1997). These are estimated from relative frequency of occurrence and relative abundance, and range from 0 (occurrence equal in groups) to 100 (present only in one group). Significance of indicator values was tested by a Monte Carlo method. The analyses were conducted using PC-ORD Version 5.0 software (McCune and Mefford, 1999).

A centred and standardized principal components analysis (PCA) was conducted for each of two matrices using districts as samples: (1) fragmentation metrics for $\geq$100 year nemoral forest and frequencies of occurrence of structural elements in nemoral tree WKHs, and (2) fragmentation indexes for $\geq$100 year nemoral forest plus $\geq$60 year aspen forest and frequencies of occurrence of structural elements in nemoral tree plus aspen WKHs. The data were relativized by maximum to avoid bias towards fragmentation indexes with high absolute levels. Significance of principal components (axes) was determined by Monte Carlo tests with 100 iterations. Spearman's rank correlation coefficients were calculated to test significant relationships of PCA axes with the fragmentation indexes and frequencies of occurrence of structural elements (habitat quality variables) and also with frequencies of species occurrence in WKH. The ordinations were performed using PCORD version 5.0, and correlation analysis using SPSS Statistics 17.0.

3. Results

Total forest area ranged from 28.6 to 55.4% among the districts and total nemoral forest area from 0.07 to 1.42%, according to data from the State Forest Register (Table 1). The relative area of nemoral forests with age $\geq$100 years was low in all districts, with the greatest area in western Latvia in the Kuldiga and Liepaja districts (0.51% and 0.41%, respectively). The relative area of nemoral forest $\geq$100 years of age plus aspen forest $\geq$60 years of age ranged from 0.13% to 0.75%

Table 3
Indicator species values in groups classified by presence of structural elements in nemoral tree WKHs and nemoral tree plus aspen (*Populus tremula*) WKH. Only statistically significant ($p < 0.05$) indicator species values are shown.

Nemoral tree WKH	Uneven-aged	Multiple decay stages	Abundant polypores	Four nemoral species	Trees with hollows	Dead standing trees
Acrocordia gemmata					9	9.9
Arthonia leucopellea	6.3	9.8	9.6		7.7	10.7
Arthonia spadicea	9.2	12.8	11.7		12.3	12
Lobaria pulmonaria	17.4	16.5	16.2	30.6	14.3	15.7
Anomodon spp.	12.1	14.0		15.9	10.1	10.8
Homalia trichomanoides	36.4	36.8	39.2	45.2	37.4	35.5
Jamesoniella autumnalis		7.5	9.0	6.2	5.3	5.7
Lejeunea cavifolia	6.2	9.3	13.1	10.6	6.8	6.1
Metzgeria furcata		6.1	6.5	8.8	5.8	5.9
Neckera complanata	10.1	10.2	10.1	12.7	9.6	9.7
Neckera pennata	27.6	33.6	32.7	40.6	24.6	29.6

Nemoral tree plus aspen WKH	Uneven-aged	Multiple decay stages	Polypores	Four nemoral species	Trees with hollows	Dead standing trees
Acrocordia gemmata					9.7	9.7
Arthonia leucopellea	6.2	7.5	6.9		6.4	8.6
Arthonia spadicea	9.1	9.2	8.0	8.6	10.1	9.5
Lobaria pulmonaria	13.8	13.1		30.8	11.5	13.2
Anomodon spp.	10.2	9.0		14.2	7.4	7.7
Homalia trichomanoides	35.3	39.1	38.1	43.6	38.3	36.4
Jamesoniella autumnalis		8.2	8.5	7.2	7.2	6.8
Lejeuneacavifolia	6.5	10.5	12.4	12	8.4	7.7
Metzgeria furcata	5.4	7.2	6.9	9.6	6.5	6.6
Neckera complanata	9.2	9.9	9.2	11.2	9.0	8.2
Neckera pennata		37.1	36.4	39.1	28.9	33.4

of the total area in a district. There was a very wide range in area and density of WKH area in the districts. For example, the relative area of WKH in districts ranged from 0.01% to 0.40% of the total land area. The relative area of nemoral forest and also nemoral forest plus aspen forest showed no significant correlation (not shown) with the respective WKH area.

Indicator species analysis showed that the studied indicator epiphyte species tended to be more common in WKH that contained the selected structural elements (Table 3). These stand-level effects of habitat quality were similar for both of the extracted data sets: nemoral WKHs and nemoral plus aspen WKHs. The highest indicator values (Table 3), which are calculated based on differences between groups in both frequency of occurrence and abundance, were found for *Homalia trichomanoides* (>35), *Neckera pennata* (>24) and *Lobaria pulmonaria* (>14).

In the ordinations of districts based on fragmentation for nemoral forests with age over 100 years plus habitat quality features in the selected WKHs, PCA axis 1 explained 51% of the variation (significant at <0.05 in 100 Monte Carlo iterations). The other PCA axes were not significant. PCA axis 1 was positively correlated to total and mean area of patches and density of patches and core area of patches, and negatively with mean distance between patches (Table 4). Most of the districts with the highest scores on PCA axis 1 (e.g. Liepaja, Talsi and Dobele and Kuldiga; Table 5) are located in western to central Latvia (Fig. 1). In this study, the frequency of occurrence of structural elements indicating better habitat quality in WKHs was associated on PCA axis 1 with poor connectivity and a decreased total area of nemoral forest that potentially could qualify as WKH. Thus, the districts with more fragmented nemoral forests tended to have better habitat quality in WKHs. These districts (e.g. Ludza, Preili, Rezekne and Jekabpils) are mostly located in eastern Latvia. Correlation of frequency of occurrence of the studied species with PCA axis 1 scores indicated that *Neckera pennata* was more common in districts that had better habitat quality in WKH, but increased fragmentation of ≥100 year nemoral forest (Table 4).

The ordination of districts based on fragmentation of nemoral forests ≥100 years plus aspen forests ≥60 years and habitat quality factors in nemoral tree plus aspen WKHs yielded two significant

Table 4
Correlation of fragmentation metrics for potential WKH forest, frequency of occurrence of structural elements and indicator epiphyte species in WKHs with PCA scores derived using matrices: (1) fragmentation metrics for ≥100 year nemoral forest and frequency of occurrence of structural elements in nemoral tree WKHs, and (2) fragmentation indexes for ≥100 year nemoral forest plus ≥60 year aspen forest and frequency of occurrence of structural elements in nemoral tree plus aspen WKHs. Nemoral tree species included are oak *Quercus robur*, ash *Fraxinus excelsior*, lime *Tilia cordata*, maple *Acer platanoides*, Wych elm *Ulmus glabra* and European white elm *Ulmus laevis*. Only statistically significant correlations are shown.

	≥100 years nemoral	≥100 years nemoral + aspen	
	PCA1	PCA1	PCA2
Fragmentation indexes			
Total patch area	0.93	−0.91	
Patch density	0.85	−0.96	
Mean patch area	0.73		−0.82
Total core area	0.98	−0.61	−0.60
Core area density	0.94	−0.83	
Mean patch core area of a patch	0.79		−0.86
Mean Euclidean distance between patches	−0.76	0.91	
WKH structural elements			
Uneven age structure	−0.46		0.51
Abundant dead wood in many decay stages	−0.49		0.64
Abundant polypores	−0.57		0.77
At least four nemoral tree species		−0.44	
Trees with hollows	−0.48		0.53
Abundant dead standing trees			0.51
WKH indicator species			
Acrocordia gemmata			
Arthonia leucopellea			
Arthonia spadicea			
Lobaria pulmonaria		−0.44	
Anomodon spp.			
Homalia trichomanoides			
Jamesoniella autumnalis			
Lejeuneacavifolia			
Metzgeria furcata			
Neckera complanata			
Neckera pennata	−0.39		0.43

Table 5
Scores of samples (districts) on PCA ordinations of two matrices: (1) fragmentation metrics for ≥100 year nemoral forest and frequency of occurrence of structural elements in nemoral tree WKHs, and (2) fragmentation indexes for ≥100 year nemoral forest plus ≥60 year aspen forest and frequency of occurrence of structural elements in nemoral tree plus aspen WKHs. Scores are ranked by value on the derived gradients. Nemoral tree species included are oak *Quercus robur*, ash *Fraxinus excelsior*, lime *Tilia cordata*, maple *Acer platanoides*, Wych elm *Ulmus glabra* and European white elm *Ulmus laevis*. Only statistically significant correlations ($p < 0.05$) are shown.

≥100 years nemoral		≥100 years nemoral + ≥60 years aspen			
District	PCA1	District	PCA1	District	PCA2
Ludza	−1.01	Madona	−0.63	Dobele	−0.78
Preili	−0.95	Limbazi	−0.59	Liepaja	−0.62
Rezekne	−0.94	Cesis	−0.57	Aizkrauke	−0.54
Jekabpils	−0.71	Ogre	−0.55	Jekabpils	−0.45
Balvi	−0.65	Saldus	−0.43	Saldus	−0.29
Aluksne	−0.61	Balvi	−0.39	Bauska	−0.29
Daugavpils	−0.49	Talsi	−0.37	Tukums	−0.23
Ogre	−0.42	Kuldiga	−0.37	Valmiera	−0.23
Valka	−0.26	Dobele	−0.37	Limbazi	−0.20
Saldus	−0.24	Liepaja	−0.23	Kuldiga	−0.17
Madona	−0.23	Aluksne	−0.22	Jelgava	−0.10
Ventspils	−0.19	Gulbene	−0.04	Gulbene	−0.08
Cesis	−0.04	Daugavpils	0.03	Aluksne	−0.01
Bauska	0.02	Aizkrauke	0.05	Talsi	−0.01
Aizkrauke	0.06	Tukums	0.15	Daugavpils	0.02
Valmiera	0.15	Valka	0.23	Balvi	0.17
Gulbene	0.31	Valmiera	0.33	Rezekne	0.20
Jelgava	0.36	Bauska	0.34	Madona	0.27
Tukums	0.53	Ludza	0.50	Ventspils	0.28
Limbazi	0.75	Preili	0.50	Ludza	0.42
Kuldiga	0.90	Rezekne	0.51	Valka	0.46
Dobele	1.01	Jelgava	0.52	Preili	0.69
Talsi	1.13	Ventspils	0.77	Ogre	0.71
Liepaja	1.55	Jekabpils	0.82	Cesis	0.77

PCA axes, explaining 31% and 26% of the total variation. PCA axis 1 was negatively correlated with total patch area and density, total core area and density, as well as the occurrence of at least four nemoral species in WKH (Table 4). This axis was positively associated with mean distance between patches (Table 4). Districts with increased connectivity and area of older nemoral and aspen forest (e.g. Madona, Limbazi, Cesis, Ogre and Saldus; Table 5) had increased frequency of occurrence of *Lobaria pulmonaria* in WKHs (Table 4). Thus, forest management needs to maintain the proportion of old nemoral and aspen forests in these districts. PCA axis 2 was positively associated with higher frequency of structural elements in WKH. However, this axis was negatively associated with mean patch area and mean core area. Apparently, better habitat quality in WKH occurred in districts that had relatively small patches of potential nemoral and aspen WKHs. On this gradient, *Neckera pennata* was more frequent in districts (e.g. Cesis, Ogre, Preili and Valka; Table 5) in which WKHs contained more structural elements. The districts Cesis and Ogre had high scores on both axes, indicating both a low level of fragmentation of older forests and high habitat quality in WKHs.

4. Discussion

At a stand level, the occurrence of the used indicator species in WKH was shown to be well associated with the presence of structural elements that are associated with minimal disturbance by humans (Table 3). Presumably, the richness of specialist species is related to habitat quality in the WKH, but this could not be estimated, as full lists of indicator species in WKHs were not made in inventory. However, habitat heterogeneity in tree diameter structure, dead wood amounts and substrate diversity are known to be associated with increased lichen and bryophyte richness (Berg et al., 2002; Gignac and Dale, 2005; Lõhmus et al., 2007; Moning et al., 2009). Unmanaged stands, compared to monocultures, have higher diversity of structural elements, which offer a wider diversity of environmental conditions and ecological niches, such as dead wood in various decay stages, large old trees, canopy gaps providing variable light conditions, multi-age and size structure of trees, and heterogenous microtopography in wet stands (Kuuluvainen, 2002). As *Lobaria pulmonaria* and *Neckera pennata* are known to have limited dispersal distances (Snäll et al., 2004, 2005), the presence of these species suggests that there has been past temporal continuity of suitable tree substrates in the WKH hosting these species. However, many of the species utilized as indicators of natural forests have long-range dispersal mechanisms, and they can easily colonise suitable habitats (Nordén and Appelqvist, 2001). Among the studied indicator species, *Neckera pennata* and *Homalia trichomanoides* showed the highest association with the presence of structural elements in stands (Table 3), as previously shown (Löbel et al., 2006a,b). *Neckera pennata* is sensitive to a high rate of habitat turnover, as colony size and age before reproduction by sexual spores (Löbel and Rydin, 2009) and distance to nearest substrate can limit dispersal (Löbel et al., 2006a,b; Snäll et al., 2004).

The present study showed that the habitat quality in nemoral WKHs in a district was associated with the level of fragmentation of ≥100-year-old nemoral forest; in districts with poorer habitat quality in WKHs, the stands that were potential WKHs were less fragmented, and vice versa (Table 4). In districts with less fragmented nemoral stands (e.g. Liepaja, Talsi, Dobele and Kuldiga), the lower amounts of structural elements in the respective WKHs indicate a legacy of removal of dead wood. In Sweden, nemoral forest WKHs were observed to contain dead wood amounts that were much lower than in natural forests that were undisturbed by man (Jönsson and Jonsson, 2007). Over time, natural disturbance will lead to increased amounts of structural elements in stands where habitat quality is presently low. A strategy of defragmentation of forests by planting in areas where connectivity is poor is that, over time, new woodland will develop features of natural forest (Quine and Watts, 2009). Therefore, a conservation aim might be to increase the area of forests set aside as WKHs in districts where habitat quality is low, by lowering the criteria used in inventory (Fig. 2). It appears that this has already been done in three districts (Dobele, Jelgava and Gulbene) that have low habitat quality

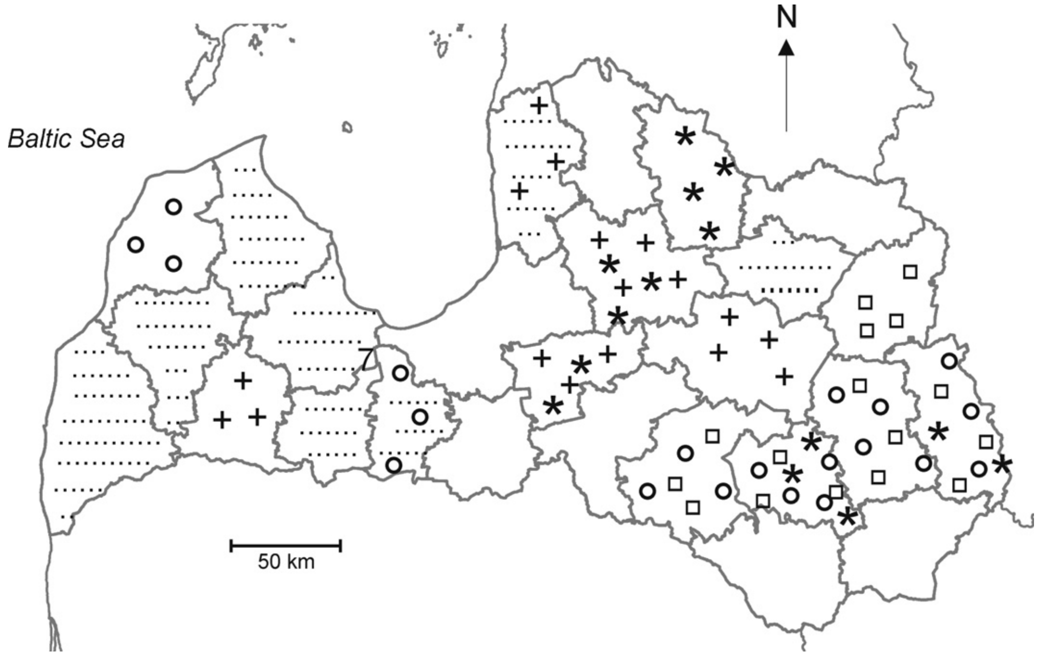

Fig. 2. Recommendations for conservation efforts in districts of Latvia: · · ·, ensure natural processes; □, increase proportion of nemoral forest protected as WKH; ○, increase the proportion of old aspen in forests; +, maintain proportion of old nemoral and aspen forests in the landscape, increase proportion protected as WKHs; *, increase patch size of old nemoral and aspen forests.

in WKH, as these districts had a larger area of WKH than the area of nemoral forest with age ≥100 years (Table 2). Thus, a part of the WKHs in these districts had an age ≤100 years. This might partly explain why there was a lack of a significant correlation between WKH area and potential WKH area. Perhaps, the persons conducting inventory in districts with generally lower habitat quality, made judgements based more on ranking of stands in the district, rather than that in the country. Thereby, the best stands available in a district were chosen as WKH. Inadvertently, this can be considered as appropriate management for conservation of biodiversity in these districts.

Districts with greater fragmentation of ≥100 year nemoral forests had greater frequencies of occurrence of structural elements and *Neckera pennata* in the WKH data set (Tables 4 and 5). This might suggest that *Neckera pennata* has survived in fragments that have retained temporal continuity. Nemoral forests in districts with increased fragmentation, all of which occur in eastern Latvia (Ludza, Preili, Rezekne, Jekabpils and Balvi), may have breaks in spatial continuity, and therefore greater risk of species loss. As these districts have a low relative area (0.01–0.06% of forests) of WKH with nemoral tree species, the WKHs in these districts need to be highlighted as potentially supporting source populations, capable of supplying diaspores to nearby stands with suitable substrate. A thorough search should be made in these areas to identify more nemoral stands for protection, and perhaps lowering the threshold for identification as WKHs (Fig. 2). The observed regional east-west difference in occurrence of *Neckera pennata* is likely not due to a climatic influence, as this species was found in all districts.

The analysis of landscape-level effects of fragmentation of nemoral forests on occurrence of indicator epiphyte species needs to consider the distribution of aspen, as this tree species is also a suitable substrate for the studied epiphytes. Aspen is a host for 200 epiphytic lichen species, of which 40 are specialists and 8 are threatened (Kouki et al., 2004). When aspen was included in the spatial model, the PCA ordination separated a connectivity and total patch area gradient (PCA axis 1) from a habitat quality and patch size gradient (PCA axis 2). Planting of new woodland increases total patch area and connectivity, but can simultaneously cause a decrease of mean patch area (Quine and Watts, 2009). However, in the present study these landscape metrics operated independently. The districts Madona, Limbazi and Cesis had a larger total patch area and increased connectivity (negative scores on PCA axis 1, Table 4). However, the proportion of ≥100 year nemoral plus ≥60 year aspen forest that was protected as WKH was very low (0.04%, Table 2) in Cesis. Districts (Jekabpils, Ventspils, Jelgava, Rezekne, Preili and Ludza) with high positive scores on PCA axis 1 (Table 5) were shown to have less connectivity and smaller total patch area of ≥100 year nemoral plus ≥60 year aspen forest. Of these districts, Preili, Rezekne, Ludza and Jekabpils were also shown to have high fragmentation of ≥100 year nemoral forest (without aspen). Except in Jekabpils, the relative area of nemoral plus aspen WKH in these districts was very low (<0.08%). The correlation of *Lobaria pulmonaria* with connectivity in the nemoral tree species plus aspen model, but not in the nemoral tree species model, suggests that aspen can compensate as a readily colonized substrate in areas where nemoral trees are in low supply. Therefore, an increase in habitat connectivity could be achieved in districts having low connectivity in a relatively short period of time by management to increase the proportion of old aspen in forests (Fig. 2), for example, by less harvest of aspen reaching 60 years, and increasing the proportion of aspen in mixed forests. However, a long time lag for recovery of species might be expected. The mean dispersal distance for *Lobaria pulmonaria* has been estimated to be 35 m over a period of 9 years, indicating a need for spatial continuity of suitable deciduous tree substrate for this species (Öckinger et al., 2005). Although *Lobaria pulmonaria* will become extinct without regeneration of host trees (Snäll et al., 2005), it is rather plastic in response to habitat change (Belinchón et al., 2009). Therefore, protection of source populations is needed, particularly in districts where *Lobaria pulmonaria* is rare.

It is known that *Neckera pennata* is restricted by dispersal (Snäll et al., 2004), but in both of the tested fragmentation data sets (nemoral forest with or without aspen), frequency of occurrence was related best with habitat quality. The Ludza, Preili and Rezekne districts, where frequency of occurrence of *Neckera pennata* was higher, suffer from both high fragmentation of nemoral forests, and fragmentation of nemoral plus aspen forests, but habitat quality of WKH is high. Presence of *Neckera pennata* is known to be associated with old aspen as a substrate (Berg et al., 2002), alongside nemoral species (Snäll et al., 2004). As for *Lobaria pulmonaria*, the spatial continuity of suitable substrate in districts where nemoral and aspen stands are in short supply should be aimed at maintaining temporal and spatial continuity of old aspen forests. Cesis and Ogre were suggested to have good connectivity and large total area of older nemoral plus aspen stands (PCA axis 1), but the mean area of the patches was small (PCA axis 2). The WKHs in these districts had high frequencies of occurrence of both *Lobaria pulmonaria* (correlation with PCA axis 1) and *Neckera pennata* (correlation with PCA axis 2). Thus, a conservation aim in these districts should be to increase the size of the WKHs that support these species (Fig. 2), by establishing buffer zones in which restoration methods to increase substrate availability are carried out.

5. Conclusions

The presence of the selected indicator species in WKHs was well explained by structural elements that improve habitat quality. However, the study identified problems in interpretation of results obtained when linking WKH data with forest inventory data. On a regional level, habitat quality was negatively associated with extent of fragmentation. Also, the WKH habitats in the study area are only a subset of the data set extracted from the forest register, i.e. the WKH represent stands with the best habitat quality in the districts. Thus, the level of fragmentation of $\geq$100-year-old nemoral forests in a district might not be related to the level of fragmentation of natural stands that meet WKH criteria. This is confirmed by the lack of significant correlation between both area and density of WKH, and area of forest that could potentially be WKH. However, linking of the two databases did provide valuable information regarding differences between districts, which could be used to identify priority conservation measures. Firstly, the WKH inventory needs to be continued, particularly in districts where incomplete. Habitat quality needs to be improved in districts with less fragmented nemoral forest (Liepaja, Dobele, Talsi and Kuldiga). This can be expected to occur in the future due to natural successional processes. The highest levels of fragmentation of both broad-laved and aspen forests occur in eastern Latvia, but habitat quality is good in the existing WKHs. Conservation efforts there need to focus on increasing the proportion of nemoral forest protected as WKH and increasing the total area and connectivity of old aspen forests.

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Effect of soil and canopy factors on vegetation of *Quercus robur* woodland in the boreo-nemoral zone: A plant-trait based approach

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ABSTRACT

The aim of the study was to determine the effect of soil and canopy on the understory vegetation of *Quercus robur* stands in Latvia, located in the boreo-nemoral zone. To determine the main processes regulating formation of the plant communities, the understory vegetation of *Q. robur* stands was described using plant traits. Vegetation and soils were described in 24 plots representing contrasting soil types and tree species composition. Redundancy analysis was used to determine the relation between vegetation, described using plant traits (proportion of species with each trait), and soil and canopy factors. About 50% of the variation in vegetation described by plant traits was explained by soil factors, which were collinear with canopy factors. Species characteristic of ancient forest were generally abundant. A smaller proportion of autochorous species, which have limited seed dispersal distances, occurred in secondary forest on previous agricultural land with higher soil pH in the upper soil horizon. A typical nemoral herb layer with greater proportion of ant-dispersed species and hemicryptophytes was associated with soils that had higher clay content. Forest management for conservation to maintain the nemoral aspect of deciduous woodland should focus on woodland that has developed on silty clay loam soils.

1. Introduction

European natural nemoral woodland is considered to be among the most degraded ecosystems in the world (Jędrzejewska et al., 1994). Throughout its former distribution range *Quercus robur* L. woodland has been severely fragmented by land-use change to agriculture, particularly on rich soils. Natural *Q. robur* woodland that has a history of no disturbance by humans has been completely lost, which has resulted in wide controversy on views of previous structure of these woods – open canopy or tall forest (Peterken, 1996; Vera, 2000; Mitchell, 2005). However, broad afforestation measures have been targeted on planting secondary *Q. robur* forests on previous agricultural lands, and in many European countries most of the *Q. robur* forest area is on former agricultural land (Brunet et al., 2011). On the other hand, in the boreo-nemoral zone forest management has often been concentrated on planting of other tree species, such as *Fagus sylvatica* L. (Lawesson, 1999) and conifers (Lindbladh et al., 2011), which has further increased fragmentation (Brunet et al., 2011).

The distribution range of *Q. robur* covers all of Europe and extends to the Ural Mountains in Russia, reaching its northern distribution range in Scotland, Sweden and Estonia (Hytteborn et al., 2005). It occurs in a vast array of forest types, including acidophilous oakwoods on oligotrophic soils, penduculate oak-hornbeam forests on rich and moderately rich soils and oak–ash forests on moist soils (European Environment Agency, 2006). In the boreo-nemoral transition region between the boreal and nemoral zones (Sjörs, 1963), *Q. robur* on rich soils forms mixed stands with nemoral tree species (*Tilia cordata* Mill.), maple (*Acer platanoides* L.), elm (*Ulmus glabra* Huds.), white elm (*Ulmus laevis* Pall.) and common ash (*Fraxinus excelsior* L.) and the boreal conifers *Pinus sylvestris* L. and *Picea abies* (L.) H. (Hytteborn et al., 2005). In appropriate soils, natural *Q. robur* regeneration is common in *P. sylvestris* stands (Dobrowolska, 2006; Goris et al., 2007).

In western Europe and southern Sweden, much is known between the relationships of understory vegetation and canopy composition in deciduous stands with former land-use, soil chemical and humus qualities, formation of a forest floor layer, humus

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accumulation rate and decomposition (Brunet et al., 1997; Verheyen et al., 1999; Verheyen and Hermy, 2001; Vesterdal, 1999; Vesterdal et al., 2012; Jandl et al., 2007; Van Calster et al., 2007; Wulf and Heinken, 2008). Tree species can affect understory community composition directly by affecting abiotic factors, such as light conditions and microclimate, and indirectly by changing the soil profile via litter (Prescott, 2010; Vesterdal and Raulund-Rasmussen, 1998; Vesterdal et al., 2008; van Oijen et al., 2005; Augusto et al., 2002; Bonifacio et al., 2008).

Classification of species based on functional plant traits has been used as a tool to understand the organization of community structure (Dupré and Erhlén, 2002; Verheyen et al., 2003; Hérault and Honnay, 2007). Floristic differences can be observed between forests developed on agriculture lands and in ancient forests, where tree cover has existed for several centuries (Verheyen and Hermy, 2004). In many regions, the ancient forests have a vital role as seed sources for dispersal of forest plants into recent forest (Honnay et al., 1998; Vellend, 2003). Ancient forest is typically defined as a woodland that has been persistent over a given period of time (Baeten et al., 2009). Lists of species that tend to occur in areas that have existed as managed or unmanaged forests for a specified long period of time have been developed in Central Europe (Honnay et al., 1998; Hermy et al., 1999). These species, called ancient forest plant species, have similar ecological profiles and strategies, low ability to colonize new territories, and lower recruitment capability (Hermy and Verheyen, 2007; Verheyen and Hermy, 2004). Moreover, persistent soil alterations caused by management change (e.g. increased phosphorus levels and acidity) can limit establishment of plant species in recent forest (Brunet et al., 1997; Verheyen et al., 1999; Wulf, 2004; Van Calster et al., 2007).

We might therefore expect that the understory vegetation of *Q. robur* woodland will differ depending on soil type, canopy species, past history of land use, habitat fragmentation and management change. To our knowledge there has been no attempt to determine the factors ruling community composition in forests in the eastern Baltic region using a plant trait approach. The landscape matrix in Latvia differs from that in Central Europe by a larger relative area of forest and dominance of coniferous trees. Considering that coniferous trees dominate in the forest landscape matrix in Latvia, we might expect (1) that the understory vegetation of mixed *Q. robur* stands is composed mostly of ubiquitous, easily dispersed boreal forest species, (2) that establishment is governed by soil factors, and that typical nemoral species with slow dispersal would likely be limited to ancient deciduous forest, in which soil profiles have not been affected by prior agricultural land use. In this study we use lack of an agricultural soil profile and presence of old trees as surrogates for identifying ancient forest. The aim of this study was to determine the effect of soil and canopy on vegetation of *Q. robur* stands, described using plant species traits and other ecological features applied at a community level.

2. Methods

2.1. Site description

Latvia is located in the transition zone of the nemoral and boreal zones (Ozenda, 1994), or the boreo-nemoral zone (Sjörs, 1963). The climate is transitional maritime to continental with mean temperature of −5.3 °C in January and 14.8 °C in July. Annual precipitation is 700–800 mm, of which about 500 mm falls in the warm period (data from the Central Statistic Bureau of Latvia). The climate is more continental towards the east. The forest area is about 55% and the dominating species are *P. sylvestris*, birch (*Betula* L. spp.) and *P. abies*, which contribute 43%, 28% and 15% of the total growing wood volume, respectively (State Forest Service, 2008). Only about 1.1% of the forest area is dominated by nemoral tree species, such as *Q. robur*.

2.2. Plots

Q. robur stands were selected from the State Forest register based on dominance of *Q. robur*, tree age >100 years and stand size >2 ha, without prior visitation. It was attempted to locate the stands regularly through Latvia, but in some areas this was not possible due to lack of *Q. robur* stands and in one case a smaller stand was chosen due to unavailability of larger stands. Size of selected stands was 1.4–34.9 ha. Upon site inspection, stands were rejected if there was clear evidence of intensive management and recent wood removal. Also, if *Q. robur* had wide canopies and other indications of recent use as wooded meadow (Vera, 2000), the stand was rejected. However, it is likely that some of the forests were previously used as woodland pasture, as this was common in the pre-1930 period (Dumpe, 1999). Location of the selected 24 stands is shown in Fig. 1. Plots with size 20 * 50 m were established in the middle of the stands. In the middle of these, in 20 * 20 m plots the projective cover as a percentage (as far as possible to the nearest 1%, but to the nearest 5% or 10% for species with high cover values) was estimated for all species in the following vegetation layers: (1). herbaceous and woody plants ⩽1 m height, (2) shrubs and saplings >1 m height, and (3) canopy and subcanopy trees. The data for 20 * 20 m plots were used for further analysis. However, an additional search for species was made in the larger plots for other species. As these always had very low cover, they were arbitrarily scored as 0.01% cover and added to the data for the 20 * 20 m plots. All trees with diameter at breast height >10 cm in the 20 * 50 m plots were cored to determine tree age. The tree cores were glued in grooved planks, sanded, and then tree rings were counted using Lignovision 1.37 (Rinntech). Maximum tree age in plots was used to determine the minimum length of time that the area had been covered by forest.

2.3. Plant traits

Twenty-five ecological features of plants were selected for the study (Table 1). These mostly included primary and derived plant traits. In addition, division by geographic distribution was chosen as it was hypothesized that soil changes with increased conifer cover in stands would lead to dominance of boreal species. For convenience, all of these ecological features are referred to subsequently as plant traits. Soil factors are known to be associated with abundance of plants grouped by Raunkiaer life form (Hérault et al., 2005) and C-S-R plant strategies (Hodgson et al., 1999). Dispersal type and vegetative spread were chosen as these traits might limit dispersal in recent stands on past agricultural land (Verheyen et al., 2003). Information on distribution, vegetative spread, seed dispersal, C-S-R plant strategies (Hodgson et al., 1999) and Raunkiaer life form (Raunkiaer, 1934) of species (Table 1) were obtained mostly from the following databases: BioPop (Poschlod et al., 2003), the Ecological Flora Database (Fitter and Peat, 1994) and Biolflor (Kühn et al., 2004). Barochorous species were combined together with autochorous species. Division was also made between ancient and other forest plant species (Hermy et al., 1999).

2.4. Soils

In each plot a pit was dug to a depth that reached the soil parent material, and the soil profile was described according to the FAO (Food and Agriculture Organization of the United Nations) WRB (World Reference Base for Soil Resources) soil classification system

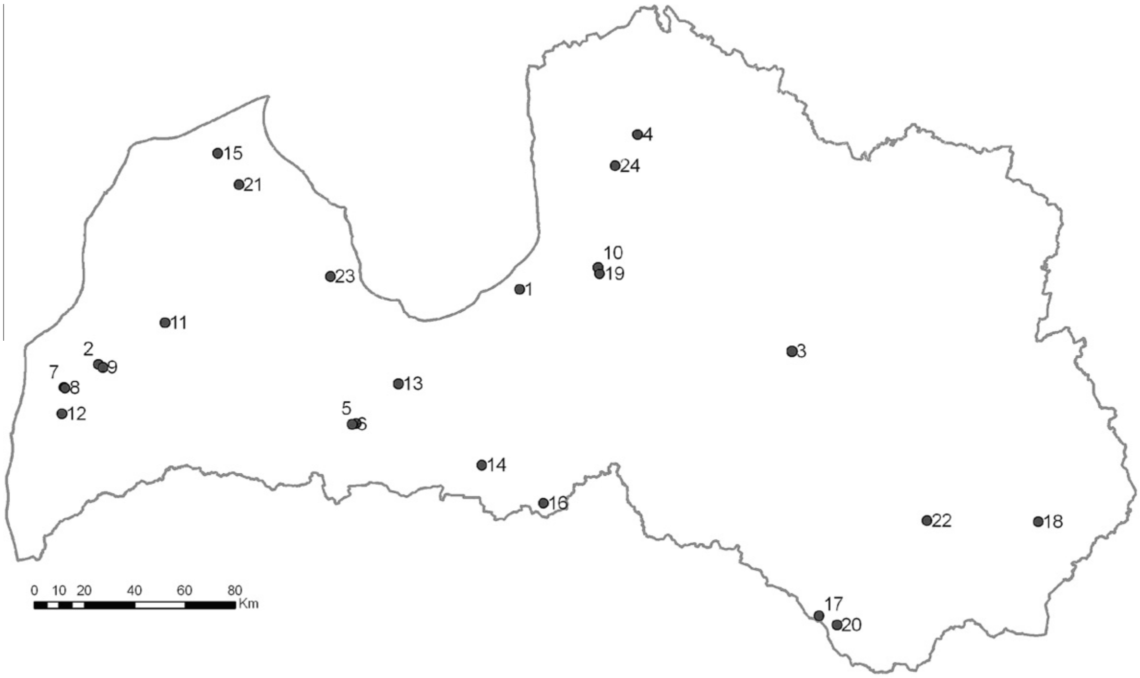

Fig. 1. Location of the studied *Q. robur* stands in Latvia.

Table 1
Distribution of species in plots by plant ecological features. Proportion of species is given as a percentage.

Plant ecological features	Proportion of species in plots (%)		
	Minimum	Maximum	Mean
Distribution			
Boreal	0	20	5
Boreal-temperate	23	57	43
Temperate	30	74	52
Vegetative spread			
Little vegetative spread	35	62	50
Short vegetative spread	8	26	15
Far vegetative spread	23	50	35
Seed dispersal			
Ants	4	28	18
Animals (excluding ants)	32	55	46
Autochorous	4	27	17
Water	0	10	2
Wind	0	41	16
Life form			
Chamaephyte	6	18	11
Geophyte	9	30	20
Helophyte	0	10	3
Hemicryptophyte	22	54	39
Phanerophyte	13	40	25
Therophyte	0	12	3
CSR strategy			
Competitor	21	48	36
Competitor-ruderal	0	10	3
Competitor–stress tolerator	9	29	19
Competitor–stress tolerator–ruderal	17	46	31
Ruderal	0	9	2
Stress tolerant	0	18	4
Stress tolerant–ruderal	0	9	2
Ancient forest plant	21	69	48

(IUSS Working Group WRB, 2007). The past use of the soils for agriculture was assessed by the presence of a plough layer (Ap), based on the following criteria: depth corresponding to a plough layer (∼30 cm) or slightly more, distinct horizon boundary (abrupt or clear) and with smooth topography. Depth to free calcium carbonates, indicated by effervescence upon application of 10% HCl, was recorded. Soil samples in 3 replicates were taken from the surface mineral topsoil horizon and the parent soil material. The replicates were pooled in the field.

The soil samples were dried at air temperature and sieved using a 2-mm mesh. Soil pH was determined with a glass electrode in 1 M KCl solution (10 g soil and 50 ml solution with three laboratory replicates). Soil fractions were determined by pipetting method (van Reeuwijk, 1995) after treatment with 0.1 M NaOH to break down aggregates. The soil fractions were defined by particle size according to the USDA soil texture classification (Cools and de Vos, 2010): sand – 0.063 to 2.0 mm, silt – 0.002 to 0.063, clay – <0.002 mm. The percentage of sand, silt and clay was calculated from fine earth (<2 mm fraction).

2.5. Redundancy analysis

In each plot the percentage of plant species in the <1 m vegetation layer with each trait (Table 1) was calculated without weighting by cover, and the data was used to create a plot-trait vegetation matrix. Proportions of species in plots were calculated to avoid a species richness effect in the analysis (species richness ranged from 11 to 38, mean = 27.8, standard deviation = 6.0). Redundancy analysis (RDA) using CANOCO version 4.53 (ter Braak and Šmilauer, 2002) was used to determine the amount of variation in plant trait composition of the herbaceous layer, which could be explained by separate sets of soil and canopy factors. The canopy factor data consisted of cover of species in the shrub/sapling layer and in the canopy/subcanopy layer. Soil factors were pH in the mineral topsoil and parent material horizons, presence of a plough layer, clay, silt and sand content in the upper horizon and parent material, and depth to free carbonates (carb), and depth of the organic layer and of the humus enriched mineral horizon. As soil and canopy factors might be collinear, the independent effect of canopy was tested by partialling out soil factors in the RDA analysis, as suggested by Borchard et al. (1992). Before the analysis, percentage data was arc-sine square transformed, as suggested for proportional data (Sokal and Rohlf, 1995; Zar, 2010), and soil variables were relativized by maximum value. Past agricultural use was used as a nominal variable. Separate RDA analyses were performed to determine the variance in plaint trait composition explained by the environmental factors, and also by canopy factors. To determine the position of species in the ordination space, a vegetation matrix composed of species cover data was used as passive data.

As constrained ordination can lead to misleading gradients in cases of relatively large number of factors with respect to sample number (McCune and Grace, 2002), the number of factors included in the final models was selected by omitting collinear variables detected for the full model using manual forward selection and by visual checks for obvious outlier effects. Forward selection using CANOCO version 4.53 is a stepwise procedure for selection of explanatory variables to be included in the model. At each step, the partial effect of each variable is tested, i.e. in addition to the variability explained by the variables already selected. In this way, the amount of variance explained was progressively increased until statistical significance of axes was obtained. In addition to that, after the forward selection procedure, the significance of each explanatory and response variable with RDA axes was tested by generalized linear models in CANOCO version 4.53 and removed if non-significant. The final models produced contained only significant variables. Thus, the final RDA ordinations contained subsets of the original response and explanatory data sets.

3. Results

3.1. Soils

The most common soil groups in the *Q. robur* stands were Luvisols, followed by Arenosols and Stagnosols (Table 2). The soil in plot 14 was strongly altered by cultivation, and a weak to strong plough layer was observed in 5 other stands (Table 2). Free carbonates were located deeper in the profiles of Arenosols (Table 2). Depth of the organic layer ranged from 0 to 42 cm. Maximum tree age in the plots was 79–418 years. The most common tree species mixed with oak in the stands was *P. abies*.

3.2. Redundancy analysis

Statistics of the redundancy analysis (RDA) are shown in (Table 3). Using vegetation matrices composed of trait variables calculated for plots, significant RDA axes were derived for variance explained by soil factors, and by canopy factors. When effect of significant soil factors (Fig. 2A) was partialled out in the RDA analysis, the RDA axes explaining joint variation with canopy factors were not significant, indicating collinearity of the canopy and soil factor data sets. Due to the lack of significance of independent variation explained by canopy factors, we do not present the results of partial RDA.

In the optimum RDA ordination that was obtained for trait variables and soil factors, GLM analysis indicated that a total of 7 soil factors were each significantly related ($p \leqslant 0.05$) to RDA axes 1 and 2 (Fig. 2A). Along the 1st axis, a deep organic horizon on sandy soils with a larger depth to free carbonates (right side of the ordination (Fig. 2A) was associated with a greater proportion of species with a boreal distribution. In these stands geophytes and a stress tolerant strategy were more common, and more species had a far creeping mode of vegetative spread. As some of the plots with a deep organic layer up to 42 cm were on wet soils (Fluvisol in plot 1 and Histosol in plot 6; Table 2), dispersal by water was also common. Typical species in these stands were *Convallaria majalis* L., *Maianthemum bifolia* (L.) F. W. Schmidt, *Polygonatum multiflorum* (L.) All., *Trientalis europaea* L. and *Vaccinium myrtillus* L. (Fig. 3). Stands on clay soils with greater relative numbers of species with a temperate distribution (e.g. *Pulmonaria officinalis* L. ssp. *obscura* (Dumort.) *Murb*, *Hepatica nobilis* Mill. and *Anemone nemorosa* L.) occurred in the ordination on the left side (Fig. 3). Greater

Table 2
Main characteristics of stands and soil profiles. Soil types are according to FAO classification system (IUSS Working Group WRB, 2007). pH of the upper horizon and clay, sand and silt content of the parent soil material are given. Tree species mixed with oak in the upper canopy (>10% cover) are listed in decreasing order of projective cover (first letters of the genus are given).

Plot	Depth to free $CaCO_3$ (cm)	Soil group	Clay (%)	Sand (%)	Silt (%)	Soil pH_{KCl}	Canopy tree species	Max. tree age (years)	Area (ha)
1	>200	Folic Gleyic Fluvisol	1.4	94.8	3.8	4.30	Alnug, Betu	211	3.1
2	66	Cutanic Calcic Luvisol	15.2	2.2	82.6	4.00	Pice, Pinu	166	8.8
3	124	Cutanic Endostagnic Albeluvisol[a]	16.4	46.4	37.2	4.40	Pice	120	1.4
4	162	Endostagnic Arenosol	9.1	32	58.9	4.60	Pice	199	8.9
5	95	Haplic Luvisol	14.9	50.8	34.3	5.20		189	7.2
6	73	Gleyic Calcic Luvic Stagnosol	28.2	2.0	69.8	5.00	Pice	110	9.5
7	73	Endogleyic Calcic Luvic Stagnosol	14.4	33.6	54.0	4.40	Pice, Betu, Frax, Popu	418	10.5
8	88	Cutanic Endostagnic Luvisol[a]	42.2	38.0	19.8	5.10	Frax	297	34.9
9	130	Endogleyic Mollic Luvic Stagnosol	16.4	36.0	47.6	4.30	Pice	172	6.5
10	110	Haplic Arenosol	3.4	90.0	6.6	4.80		194	11.0
11	130	Endogleyic Arenosol[a]	42.1	50.0	7.9	4.30		215	4.1
12	62	Cutanic Endogleyic Endostagnic Calcic Luvisol	36.1	1.4	62.5	4.30	Betu	113	5.6
13	66	Histic Calcic Gleysol	29.4	0.4	70.2	7.40	Alnug, Frax	191	12.3
14	67	Endogleyic Calcic Phaeozem[a]	7.5	29.8	62.7	7.00	Frax	240	17.9
15	>230	Endostagnic Arenosol[a]	2.7	82.6	14.7	4.10	Betu	79	5.2
16	90	Sapric Ombric Rheic Histosol	9.8	38.0	52.2	4.20	Pice, Betu	103	6.7
17	60	Endostagnic Calcic Luvisol	25.8	1.2	73.0	4.50	Pice	196	2.9
18	39	Endostagnic Calcic Luvisol	27.0	12.2	60.8	5.10	Popu	213	7.6
19	>200	Haplic Arenosols	8.7	79.8	11.4	4.50	Pice	208	3.8
20	68	Calcic Luvisol	25.1	23.1	51.7	4.50	Pice, Betu,	92	4.3
21	180	Cutanic Stagnic Luvisol	17.9	61.4	20.7	4.40	Pice	184	5.6
22	160	Endogleyic Luvic Stagnosol[a]	16.5	50.0	33.5	4.50	Pice	173	4.1
23	66	Endogleyic Calcic Luvic Stagnosol	7.1	62.4	30.5	4.40	Popu, Betu, Frax	186	21.4
24	58	Cutanic Endostagnic Calcic Luvisol[a]	28.9	1.8	69.3	5.80	Pice	268	6.4

[a] Evidence of a plough layer.

Table 3
Redundancy analysis (RDA) statistics. The response data matrix consisted of proportions of species grouped by plant traits in plots. Two explanatory matrices were used: (1) soil factors and (2) overstory plus shrub layer factors. MonteCarlo test statistics and the amount of variance explained by RDA axes are given.

		Traits (% species) and soil factors	Traits (% species) and overstorey + shrub layer factors
Monte Carlo test			
1st RDA axis	λ-value	0.18	0.18
	F	2.89	2.59
	p	0.01	0.03
All RDA axes	λ-value	0.50	0.62
	F	1.28	1.77
	p	0.05	<0.01
% Variance explained			
1st RDA axis		36.6	28.8
1st + 2nd RDA axis		53.4	49.5

proportions of species in these plots were dispersed by ants (e.g. *P. officinalis* and *Aegopodium podagraria* L.) and were hemicryptophytes (e.g. *Ranunculus cassubicus* L. and *H. nobilis*).

The 2nd RDA axis separated plots with evidence of a plough horizon, which generally had a higher pH. Plots on the lower side of the ordination had a higher silt content. The association of autochorous plus barochorous species with soils having a lower silt content was rather caused by a low relative number of these species (e.g. *Oxalis acetosella* L.) in plots with past agricultural use. The <1 m vegetation layer on agricultural soils typically included woody species, such as *Q. robur*, *F. excelsior* and *Padus avium* Mill (Fig. 3). The proportion of ancient forest plant species in the plots ranged from 21% to 69%, with a mean of 48%, but this response factor lacked significant correlation with ordination axes and was not included in the final model.

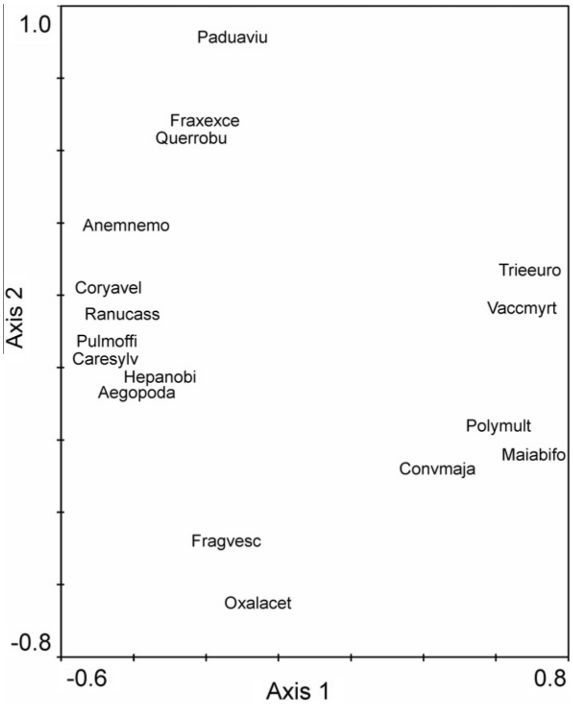

Fig. 3. Vegetation data used as passive data in a redundancy analysis ordination of a response matrix composed of proportions of plants in plots grouped by plant traits and a predictor matrix containing soil parameters. To ease readability, only the species mentioned in the text are shown. Species acronyms are given, which are composed of the first four letters of the genus and species.

The RDA ordination of plot-trait variables with canopy factors showed practically the same vegetation gradients as with soil factors (not shown), confirming collinearity of the two explanatory

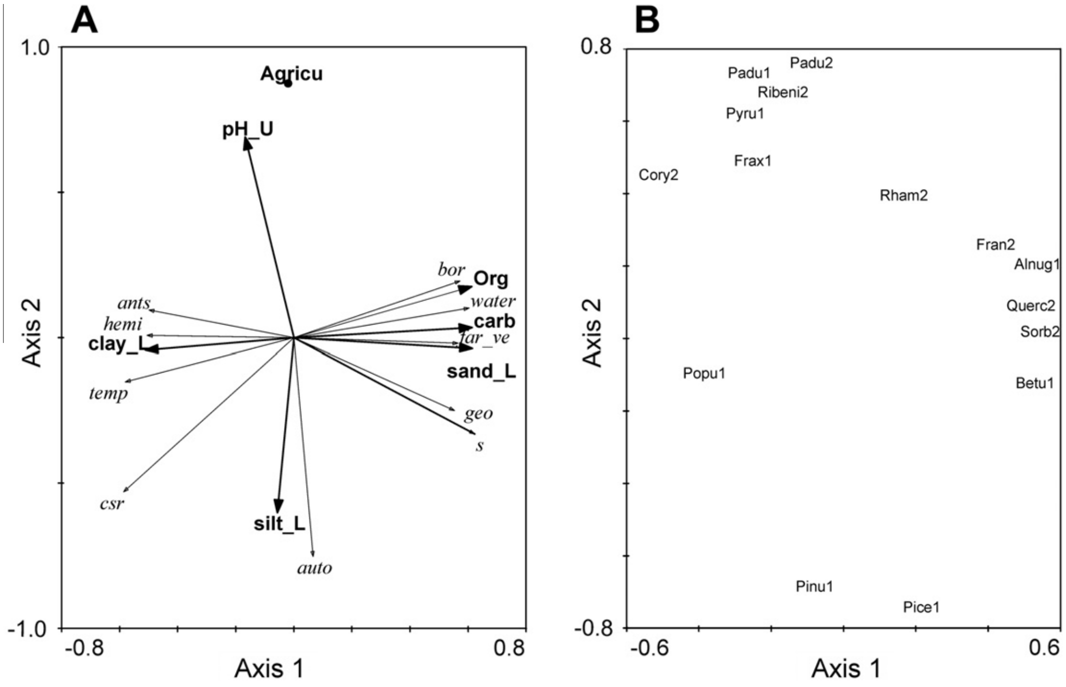

Fig. 2. Redundancy analysis ordination of (A) a response matrix composed of data on proportions of plants in plots grouped by plant traits and a predictor matrix containing soil parameters. (B) Canopy and shrub/sapling data used as passive data in the ordination shown in A. Numbers after shrub and canopy species acronyms refer to 1 – canopy layer and 2 – shrub/sapling layer. Species acronyms are given, which are composed of the first four or five letters of the genus. Only statistically significant soil variables and plant traits characteristics are shown. Soil variables: pH in the mineral topsoil horizon (pH_U), plough horizon (Agricu), clay, silt and sand content in the parent material, depth to free carbonates (carb) and depth of organic layer. Plant traits: temperate (temp) and boreal (bor) distribution, s and csr plant strategies, autochorous (auto), water and ant-dispersal of seeds, geophyte (geo) and hemicryptophyte life forms, and a far vegetative (far_ve) means of spreading.

data sets. Therefore, we show the canopy factors as passive variables in the ordination of plot-trait variables with soil factors (Fig. 2B). In the ordination, mixed *Q. robur* stands with *Alnus glutinosa* (L.) Gaertn. and *Betula pendula* Roth in the canopy and *Frangula alnus* Mill., *Q. robur* and *Sorbus aucuparia* L. in the shrub layer occurred on the right side of the ordination. *Q. robur* stands on clay soils with low cover of other woody species in the canopy were located on the left side of the ordination. The plots on agricultural soils (top of the ordination) had a denser shrub layer with *P. avium* and *Ribes nigrum* L., while *Q. robur* stands with *P. abies* were more common on silty soils (bottom of ordination).

4. Discussion

4.1. Soil types

The range of soil types in the studied *Q. robur* stands indicates a fairly wide edaphic niche, as is typical in its range (Jones, 1959). The large amount of variation in the vegetation data set explained by soil factors indicates that, to a large degree, edaphic conditions in the *Q. robur* stands formed a template for determining the species that could establish in the understory. The distribution of species with different life histories has been found to be associated with soil factors, such as soil pH, nitrogen mineralization, and amount of organic material in soil (Dupré and Erhlén, 2002; van Oijen et al., 2005). In our study, wet soils with a deep organic layer had a greater proportion of geophytes and species with far-creeping modes of vegetative spread. Many of these, such as *T. europaea* and *Maianthemum bifolium*, are ubiquitous in the boreo-nemoral zone in deciduous and coniferous forest (Taylor et al., 2002; Caimi, 2008). Accumulation of organic material is a characteristic of northern ecosystems (Vesterdal and Raulund-Rasmussen, 1998; Prescott et al., 2000; Bonifacio et al., 2008), and hence may explain the increased proportion of boreal species, such as *V. myrtillus*. The tree (*A. glutinosa*, *S. aucuparia* and *B. pendula*) and shrub (*F. alnus*) species in these *Q. robur* stands also are common elements of wet deciduous woodland in the boreal zone (Hytteborn et al., 2005).

4.2. Ancient forest species

Clearly habitat fragmentation has not limited colonization of many boreal species included in the ancient forest species lists. Considering the maximum tree ages in the stands, which in most was over 150 years, a large part of the stands could be classified as ancient forest. However, soil profiles indicated that some of the stands had past agricultural use. These stands had lower proportions of autochorous species, but they did not differ from the other stands in the proportion of ancient forest species, which reached even 50% of the total species number. Honnay et al. (1998) estimated that if 25–27 species are ancient forest species, then there is a 75% of correct identification of a stand as an ancient forest. In our study, the proportions of ancient forest species in the plots imply that the age of the studied stands has been sufficient to allow some recovery of specialist forest species, even in those with a visible plough layer. In the early 19th century, von Löwis (1824) considered that most of the mid-aged *Q. robur* stands in the area of north-eastern Latvia had regenerated from natural succession on slash and burn cultivated land that had been abandoned, while some areas had been planted by large landowners. This supports the idea of sufficient time for recovery from former agricultural land use.

4.3. Boreal features

The ordination indicated that mixed *Q. robur* stands with larger cover of the boreal conifers *P. abies* and *P. sylvestris* occurred on mesic stands with higher silt content (Fig. 2b). The lower pH in these stands can be explained by the strong acidifying effect of coniferous litter (Augusto et al., 2002; Hagen-Thorn et al., 2004). *P. abies* prefers fine textured soils (Levula et al., 2003), as suggested by the RDA ordination (Fig. 2), but was present in the canopy of most stands (Table 2), and missing or in low density in only 5 of the 24 stands, which tended to have higher pH (Fig. 2b). Typical species in stands with *P. abies* were *Frageria vesca* and *O. acetosella*. The latter has poor seed production (Dupré and Erhlén, 2002) and a ballistic means of seed dispersal, which impose limits on colonization (Berg, 2000). Therefore, it is interesting that *O. acetosella* is one of the most common herbs in *P. abies* forest, it is used as an indicator of mesic soils in the Latvian forest classification (Bušs, 1981) and appears on post-agricultural soils almost immediately with formation of a spruce canopy (Gutko et al., 2001), but also is classified as an ancient forest plant. This might imply that *O. acetosella* is a poor indicator of ancient forest in boreo-nemoral Europe, as there is regional variation in suitability of a plant as an ancient forest plant (Hermy et al., 1999).

4.4. Nemoral features

A typical nemoral herbaceous layer with *P. officinalis*, *H. nobilis* and *A. podagraria* and shrub layer with *Corylus avellana* L. occurred in *Q. robur* stands on soils with a higher clay content. Most of the soils in plots with a higher clay content (Table 2) can be classified by texture as silty clay loams (also clay loam, silt loam and loam) based on the proportions of clay, sand and silt. In these stands, seed dispersal by ants had greater importance for a larger proportion of herbs (e.g. *A. nemorosa*, *R. cassubicus*, *P. officinalis* and *H. nobilis*). Due to the rather low dispersal distances of seeds by ants, up to a maximum distance of about 10 m (Ness et al., 2004), a high relative proportion of ant-dispersed species in a stand can indicate that it is an ancient forest (Brunet and von Oheimb, 1998a; Honnay et al., 1998) or that fragmentation of deciduous forest has not limited dispersal of these species. Post-agricultural invasion of specialist forest species occurs faster in stands that are located close to an ancient forest (Brunet et al., 2011). Our study indicates that in the Baltic region there is an urgent need to determine the effects of past and present habitat fragmentation and land-use on plant communities in nemoral woodland, as this information is generally lacking.

4.5. Conservation

In conservation of nemoral forest in Latvia, there has been increasing focus in management to reduce encroachment of boreal tree species in post-agricultural nemoral woodland, particularly of *P. abies* by selective cutting and tending (Latvian Fund for Nature, 2008). The present study indicates that nemoral vegetation in *Q. robur* stands was associated with silty clay loam soils. In the long-term, if these stands were invaded by *P. abies*, continuous deposition of spruce litter might change soil characteristics, such as increased accumulation of organic matter (Bonifacio et al., 2008), lower pH, and increase shading, which are soil characteristics known to be unfavoured by nemoral species (Brunet et al., 2011). For example, colonization of *A. nemorosa* in recent woodland was observed to be impeded by low soil pH and high canopy shading (Brunet and von Oheimb, 1998b). There is some evidence that in mixed woods the presence of broadleaved tree species can retard the podzolization process (Bonifacio et al., 2008). Nevertheless, in order to maximize number of forest specialist species, management to maintain a patch shrub layer and canopy cover of 80% has been recommended in southern Sweden (Brunet et al., 2011). We suggest that forest management for conservation to maintain the nemoral aspect of deciduous woodland should focus on woodland that has developed on silty clay loam soils. However, in the boreo-nemoral zone, *P. abies* will naturally invade *Q. robur* stands, and management by cutting *P. abies* will be creating an "artificial" woodland, perhaps resembling traditional forest pasture (Vera, 2000). Therefore, in conservation of *Q. robur*, the conflict between aims of creating protected areas set-aside for natural succession and maintaining traditional management practices in woodland needs to be resolved.

5. Conclusions

In the studied stands soil factors were associated with canopy composition, and both soil and canopy factors explained about 50% of the variation in vegetation described by plant traits. Species characteristic of ancient forest were generally abundant, indicating that the stands had at least existed for a long period of time since agricultural use. However, fewer autochorous species occurred in recent forest on previous agricultural land with higher soil pH in the upper soil horizon, which might be due to dispersal limitation or failure to establish on inappropriate soils. A typical nemoral herb layer with greater proportion of ant-dispersed species and hemicryptophytes was associated with soils that had higher clay content.

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Complete Genome Sequence of the Enterobacter cancerogenus Bacteriophage Enc34

Andris Kazaks, Andris Dislers, Gerd Lipowsky, Vizma Nikolajeva and Kaspars Tars

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Complete Genome Sequence of the *Enterobacter cancerogenus* Bacteriophage Enc34

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***Enterobacter cancerogenus* is widely distributed in nature and is generally recovered from environmental or vegetal sources. In some cases, it has also been associated with human infections. In this study, the complete genomic sequence of virulent *E. cancerogenus* bacteriophage Enc34 was determined. The Enc34 genome is 60,364 bp in length and contains 80 open reading frames. To our knowledge, this is the first report of a bacteriophage infecting *E. cancerogenus*.**

Bacteriophages are very common organisms in all natural environments. Due to their highly specific host recognition, bacteriophages have potential as therapeutic agents in the treatment of certain human and plant bacterial infections (3, 6) and as useful diagnostic tools (12). *Enterobacter cancerogenus* (synonym, *Enterobacter taylorae*) (8) is a facultatively anaerobic Gram-negative bacterium that is generally recovered from environmental or vegetal sources and is considered mostly phytopathogenic (1). It is also a naturally occurring human gut symbiont that is known to exhibit resistance to certain antibiotics, such as aminopenicillins and cephalosporins (13). *E. cancerogenus* can cause infections of the bones and joints associated with severe trauma or crush injuries (2, 9). Here, we report the full genome sequence and organization of novel virulent bacteriophage Enc34, which infects *E. cancerogenus*.

Bacteriophage Enc34 was purified from *E. cancerogenus* lysate by ultracentrifugation in a cesium chloride gradient. Morphologically, bacteriophage Enc34 has an icosahedral head and long, noncontractile tail, which indicate that it belongs to the family *Siphoviridae* (4). The genomic DNA of Enc34 was isolated by proteinase K treatment and phenol-chloroform extraction. Genome sequencing was performed by primer walking at Microsynth in Switzerland. Putative open reading frames (ORFs) were identified by GeneMark.hmm (10) and GeneMarkS (7) software. The translated ORF products were compared with known protein sequences using BLASTP (5) and the nonredundant public GenBank database. The tRNA sequences were analyzed using the tRNAscan-SE server (11).

The genomic sequence of bacteriophage Enc34 contains 60,364 bp of linear double-stranded DNA with a G+C content of 51.07%. A similarity search for homologous genome sequences found only 42% query coverage (displaying 73 to 79% identity) with *Staphylococcus* phage SA1 (GenBank accession number, GU169904); however, the SA1 genome comprises 147,303 bp, which is 2.4 times larger than the Enc34 genome. A total of 80 putative ORFs were detected, with the shortest containing 60 amino acids. Seventy-five of the proposed genes had ATG initiation codons, four began with GTG, and one began with TTG. The genes were organized into functional clusters encoding proteins for (i) DNA replication and packaging, (ii) head and tail morphogenesis, (iii) host lysis, and (iv) DNA regulation and modification. No tRNA genes were found. Forty-five of the ORFs were annotated as hypothetical proteins. The DNA replication and packaging module contained genes encoding a DNA primase, DNA polymerase and DNA helicase, as well as small and large terminase subunits and the phage portal protein. Three structural proteins were encoded in the head morphogenesis module, and one of these proteins was predicted to be processed by the prohead protease. As proposed for other bacteriophages with Gram-negative hosts, the lysis module included the holin, endolysin, Rz, and Rz1 proteins arranged in the canonical order.

In conclusion, to our knowledge, this is the first report of a bacteriophage that is lytic for *E. cancerogenus*. Further experiments will address the detailed study of phage head assembly and lysis mechanisms.

Nucleotide sequence accession number. The complete genome sequence of bacteriophage Enc34 is available in GenBank under accession number JQ340774.

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Chapter 20
Increased Auxin Content and Altered Auxin Response in Barley Necrotic Mutant *nec1*

Anete Keisa, Ilva Nakurte, Laura Kunga, Liga Kale, and Nils Rostoks

Abstract The role of hormone crosstalk in plant immunity is lately emerging as significant topic of plant physiology. Although crosstalk between salicylic acid and auxin affects plant disease resistance, molecular mechanisms of this process have not yet been uncovered in details. Mutations disrupting cyclic nucleotide-gated ion channel 4 (CNGC4) affect SA-mediated disease resistance in barley *Hordeum vulgare* and in *A. thaliana*. Significantly, decreased stomatal apertures of barley CNGC4 mutant *nec1* and dwarfed stature of *A. thaliana* CNGC4 mutant *dnd2* suggest that nonfunctional CNGC4 might be affecting also auxin signaling. Excised coleoptile elongation, stomatal conductance, and cell size measurements assaying physiological effect of exogenous auxin treatment suggested altered auxin signaling in *nec1* mutant. Real-time qPCR analysis identified significant change in mRNA abundance of four auxin-related genes – *YUCCA1, VT2, HVP1*, and *TIR1*. Analysis of endogenous auxin content of *nec1* plants detected ca. fourfold increase in indole acetic acid (IAA) content in *nec1* leaves and roots compared to wt plants, as measured by HPLC. These results suggest that apart from SA-related disease resistance, CNGC4 functions also in auxin signaling in barley; therefore, barley *nec1* mutant could serve as model system revealing role of SA-auxin crosstalk in plant disease resistance.

Keywords Barley • *nec1* mutant • Lesion mimic mutant • Auxin • Disease resistance

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20.1 Introduction

Hormone crosstalk in plant immunity is lately emerging as significant topic of plant physiology (Spoel and Dong 2008; Pieterse et al. 2009; Grant and Jones 2009; Verhage et al. 2010). Although well-known antagonistic effect of salicylic acid (SA) and jasmonate/ethylene signaling is certainly a central backbone of hormonal control of plant immunity (Glazebrook 2005), several other phytohormones feed into this central signaling pathway. Auxin has previously been mainly associated with plant growth and development, but recently, it has become apparent that it also plays a significant role in plant immunity (Spaepen and Vanderleyden 2011). For example, resistance of *A. thaliana* to virulent bacteria *Pseudomonas syringae* pv. *tomato* DC3000 (Pst DC3000) requires suppression of auxin signaling, and exogenously applied auxins render *A. thaliana* more susceptible to infection (Navarro et al. 2006). AvrRpt2 alters host's auxin signaling to ensure virulence on *A. thaliana* lacking corresponding R-gene (Chen et al. 2007). Limited evidence suggests that crosstalk between auxin signaling and immunity is operating also in monocots. For example, constitutive expression of auxin-conjugating enzyme GH3-8 in rice promotes resistance against *Xanthomonas oryzae* pv. *oryzae* (Ding et al. 2008) and over-expression of OsWRKY31 enhances resistance to *Magnaporthe grisea* and altered auxin responsiveness of rice. In addition, SGT1, a protein required for auxin signaling and disease resistance in *A. thaliana* (Azevedo et al. 2006), also participates in auxin signaling and disease resistance of rice (Wang et al. 2008). SGT1 is also required for certain R-gene mediated powdery mildew resistance in barley (Azevedo et al. 2002).

Repression of auxin signaling is thought to be a common mechanism employed by SA-mediated disease resistance (Wang et al. 2007). Auxin interferes with *PR*-gene up-regulation in response to SA (Iglesias et al. 2011) and vice versa – SA represses auxin receptors and thereby stabilizes auxin signaling repressors (Wang et al. 2007). Despite the evidence supporting antagonistic effect of auxin and plant disease resistance, auxin signaling components have recently been shown to participate in systemic acquired resistance (Truman et al. 2010) indicating a complexity of mechanisms underlying SA-auxin crosstalk.

SA-auxin crosstalk is also supported by the fact that several plant mutants exhibiting altered disease resistance and overaccumulating SA display phenotype resembling distorted auxin signaling (Wang et al. 2007). Therefore, SA-overaccumulating mutants can help to advance our knowledge of role of SA-auxin crosstalk in plant immunity. Mutations disrupting cyclic nucleotide-gated ion channel 4 (CNGC4) affect SA-mediated signaling and plant resistance in barley (Keisa et al. 2011) and in *A. thaliana* (Balague et al. 2003; Jurkowski et al. 2004). Barley necrotic mutant *nec1* containing defective CNGC4 exhibits increased concentration of SA and H_2O_2 (Keisa et al. 2011). In this study, we show that *nec1* mutation also affects auxin signaling in barley supporting the role of crosstalk between SA and auxin in monocot immunity.

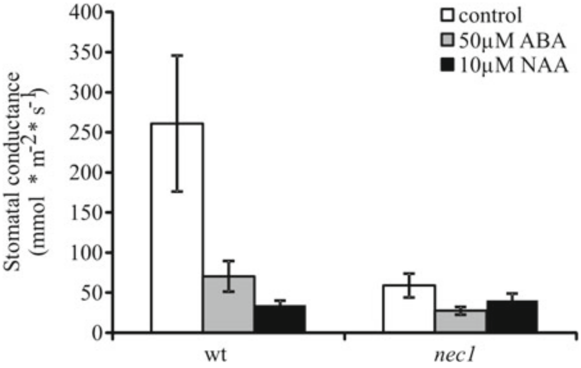

Fig. 20.1 Stomata of *nec1* and parental cv. "Parkland" respond differentially to synthetic auxin 1-naphthalene acetic acid (NAA). Plants were pretreated with 50 µM abscisic acid (ABA) and after that subjected to treatment with 10 µM NAA. Error bars represent the standard deviation of means

20.2 Materials and Methods

20.2.1 Plant Material

All experiments were conducted with barley necrotic mutant GSHO 1284 (further in text – *nec1*) carrying mutant allele *nec1.c*. *nec1.c* allele contains an insertion of a transposable element within an intron of the gene that causes alternative splicing and predicted nonfunctional protein coding sequence (Rostoks et al. 2006). GSHO 1284 is essentially isogenic to its parental variety Parkland (Keisa et al. 2011).

20.2.2 Stomatal Response to Exogenous Auxin

Stomatal responses to exogenous abscisic acid and 1-naphthalene acetic acid (NAA) were determined by measuring stomatal conductance using Li-6400 Portable Photosynthesis System (Li-Cor Inc., USA). Second leaves from 20 plants (five plants per pot) from Parkland as well as *nec1* were used for measurements. MES-KCl buffer (Bright et al. 2006) was sprayed several times on 2-week-old plants to open stomata. After 4 h, stomatal conductance was measured first time (Fig. 20.1, control). Then plants were sprayed with MES + 50 µM ABA solution and measured again. Third measurement was performed after plants were sprayed with MES + 50 µM ABA + 10 µM NAA solution (Fig. 20.1).

20.2.3 Coleoptile Elongation in Response to NAA

Coleoptile elongation was assayed as described by Kotake et al. (2000) with minor modifications. Briefly, 5-mm-long coleoptile segments (3 mm below the tip off the

Table 20.1 Genes and corresponding primers used for qRT-PCR analysis

Gene/harvest 35 unigene	*A. thaliana* homologue	Primer sequence 5′–3′
YUCCA1	*AtYUC1*	F:ATGGAGGTCTCCCTGGACCTGT
6837	AT4G32540	R:TCACCTTGTCCACGAACCAGAG
VT2	*AtTAA1*	F:GTATCCTGCCGTGACGGACTTC
11280	AT1G70560	R:ACTGCGGCCAGTAGTAGGCAAG
NIT2	*AtNIT1*	F:GTACCTGGGTAAGCACCGCAAG
17606	AT3G44310	R:GTGCTGTCCTTAACAGTGGCATC
HVP1	*H^+PPase*	F:AGCCACAGAATTCGTGAAAGAACTG
534	AT1G15690	R:GCCCAATAAAGACATTAGGTGTCAGG
H^+-ATP	*H^+ATPase*	F:TCTGGCTCTTCAGCATTGTG
14211	AT4G30190	R:ACGGTAGCTGCTCTTGTCGT
TIR1	*AtTIR1*	F:TGCCAAGCTGGAGACAATGC
2793	AT3G62980	R:CGGGCTATCATCCGGAAGTG
SGT1	*AtSGT1a*	F:TCTGGTGACTCAAGGTTTACTCGTC
1570	AT4G23570	R:GTGCATTCTCCATATTTTCACCATC
ABP1	*AtABP1*	F:TCCTGTCGCTCAGAATAGCA
19159	AT4G02980	R:CCAGACAAAGGGGAACTTCA
GAPDH		F:CGTTCATCACCACCGACTAC
19159		R:CAGCCTTGTCCTTGTCAGTG

seedling) were excised from 3-day-old barley seedlings grown in dark at 22°C. Excised segments were floated in dark for 4 h in 5 μM NAA solution containing 5 mM KCl, 50 μM $CaCl_2$, and 10 mM MES, pH 6.5.

20.2.4 Gene Expression Analysis Using Real-Time qRT-PCR

For RNA extraction, segments of cotyledon leaf from two-week-old plants of necrotic mutant *nec1* and parental cv. RNA extraction, cDNA synthesis, and quantitative real-time PCR were performed as described in Keisa et al. (2011). Primers used for qRT-PCR analysis are listed in Table 20.1. Relative quantification was performed using $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001). Transcript levels of the studied genes were normalized to *HvGAPDH* transcript value in the same sample.

20.2.5 IAA Detection and Quantification Using HPLC

For leaf tissue samples, seedlings were grown in soil at 22°C under long-day (16 h day, 8 h night), medium-light (ca. 150 μmol m^{-2} s^{-1}) conditions. For root samples, plants were hydroponically grown in Knop's salt solution at 22°C under long-day (16 h day, 8 h night) conditions. Leaf and root samples were taken from 14-day-old plants.

Extraction and purification for auxin was performed as described by (Dobrev and Kaminek 2002) with minor modifications (Nakurte et al. unpublished). Briefly, the plant material was ground in liquid nitrogen and extracted with 100% methanol. Samples were pre-concentrated by SPE using AccuBOND II ODS-C18 200 mg 3 ml SPE (Agilent). Chromatographic analysis was performed on a modular HPLC system, Agilent 1100 series (Agilent Technologies, Germany). HPLC separations were achieved by using a reverse-phase Zorbax Eclipse XDB-C8 (Agilent Technologies, Germany) column 4.6 × 150 mm, 5 μm. Mobile phase composed of methanol and 1% acetic acid (60:40 $v\,v^{-1}$) in isocratic mode at a flow rate of 1 ml min^{-1}. The detection was monitored at 282 nm (Ex) 360 nm (Em) (IAA, IPA). The developed method was validated in terms of accuracy, precision, linearity, limit of detection, limit of quantification, and robustness. Standards of indole-3-acetic acid (>99%) and indole-3-pyruvic acid (99%) were purchased from Sigma-Aldrich (St. Louis, USA).

20.3 Results

20.3.1 nec1 Phenotype Suggests Changes in Auxin Signaling

We performed several physiological auxin sensitivity tests to assay *nec1* auxin response. Since auxin is one of the regulators of stomatal conductance, we measured stomatal conductance of *nec1* and parental line Parkland. *nec1* mutant showed significantly reduced stomatal conductance at otherwise permissive conditions. This prompted us to examine *nec1* stomatal response to exogenously applied synthetic auxin 1-naphthalene acetic acid (NAA). Since auxin is known to inhibit abscisic acid (ABA)-induced stomatal closure (Tanaka et al. 2006), we examined NAA effect on ABA-treated barley leaves. Further reduction of stomatal conductance in *nec1* plants was observed suggesting that *nec1* plants respond to ABA. In contrast to ABA response, *nec1* showed significantly different response to NAA compared to wt plants, that is, NAA reversed the ABA-induced stomatal closure in *nec1* plants, but closed them even further in cv. Parkland (Fig. 20.1).

We also examined three physiological responses traditionally serving as indicators for altered auxin response – root gravitropic response, cell size, and coleoptile elongation. Endogenous auxin regulates cell expansion (Perrot-Rechenmann 2010). We used leaf impression method (Khazaie et al. 2011) to determine epidermal cell size. Epidermal cells from middle part of adaxial surface were measured. Unlike *dnd2* in *A. thaliana*, *nec1* does not exhibit dwarfed stature. However, average perimeter of epidermal cells in *nec1* was somewhat shifted toward lower values (Fig. 20.2a). Root tip curving in response to gravistimulation at 90° to the vertical was not significantly altered in *nec1* (Fig. 20.2b). Coleoptile elongation is one of the tests routinely used for characterizing auxin sensitivity in plants. Exogenously applied auxin triggers excised coleoptile elongation in barley in dose-dependent manner (Kotake et al. 2000). Detached coleoptile test showed differential response to NAA in *nec1* plants as the *nec1* coleoptiles showed higher increase in length following application of exogenous auxin (Fig. 20.2c).

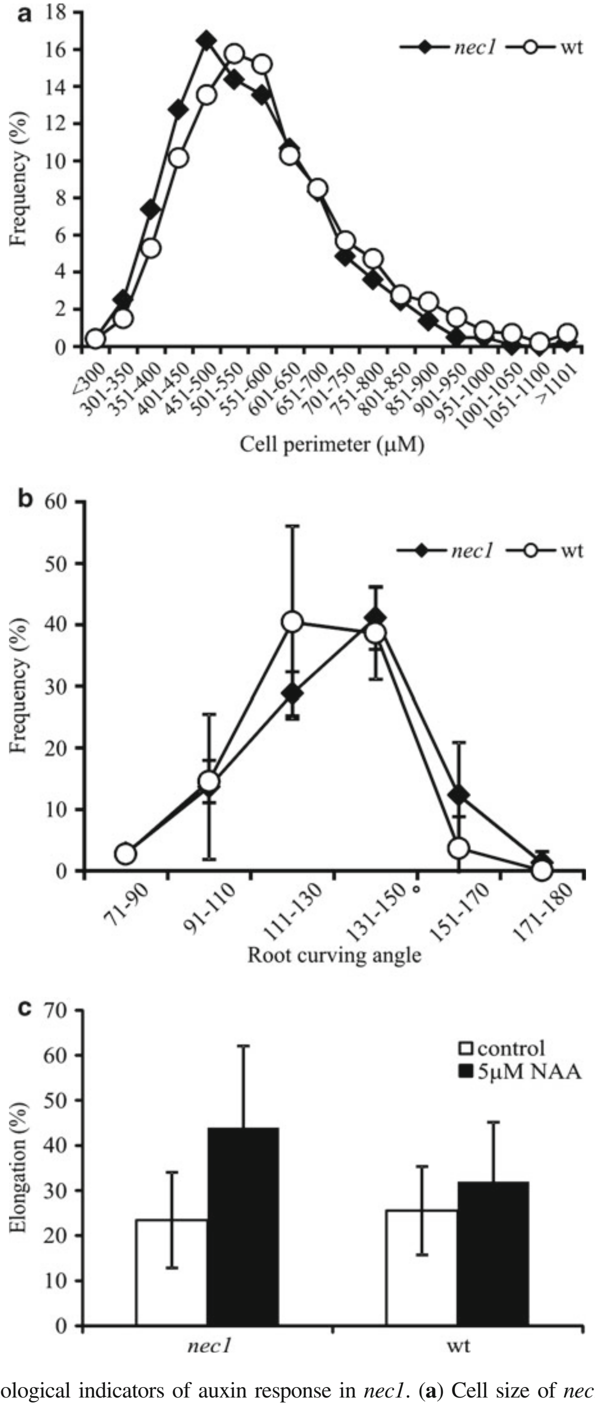

Fig. 20.2 Physiological indicators of auxin response in *nec1*. (**a**) Cell size of *nec1* and wt plants. Perimeter of epidermal cells ($n > 1{,}500$) from 10 plants from *nec1* and wt was measured. (**b**) Angle of root curvature after gravistimulation at 90° to the vertical. Root gravitropic response of *nec1* and wt does not differ significantly. Seedlings were grown for 3 days vertically and then rotated by 90°. Error bars represent the standard deviation of means ($n = 20$ in each – *nec1* and wt). (**c**) Exogenously applied NAA differentially affects coleoptile elongation in *nec1* and wt plants. Results are expressed as percent increase compared to initial segment length. Error bars represent the standard deviation of means

20.3.2 nec1 Mutation Affects Expression of Auxin Signaling Related Genes

To gain a better understanding of molecular basis of the observed differences in *nec1* physiological auxin response, the expression of several key genes involved in auxin biosynthesis, perception, and downstream signaling was studied using quantitative real-time PCR.

SGT1 genes have been identified as enhancers of the auxin response defect conferred by the *tir1-1* mutation, and the SGT1b protein was shown to be required for the $\mathrm{SCF}^{\mathrm{TIR1}}$-mediated degradation of Aux/IAA proteins (Gray et al. 2003). *SGT1* is also required for *MLA*-mediated barley resistance to powdery mildew pathogen *Blumeria graminis* f.sp. *hordei* (Azevedo et al. 2002, 2006). ABP1 (auxin-binding protein 1) was the first protein that was proved to bind auxin directly (Hesse et al. 1989; Teale et al. 2006), although recent discovery of the TIR1 as the auxin receptor (Dharmasiri et al. 2005; Kepinski and Leyser 2005) suggests that it may not be its primary function. Nonetheless, several recent studies suggest it is essential for auxin perception and response (Braun et al. 2008; David et al. 2007; Effendi et al. 2011; Tromas et al. 2009). Moreover, ABP1 has been implicated in stomatal regulation (Gehring et al. 1998). *H^+-ATPase* encodes a plasma membrane proton pump that is dephosphorylated through an ABA-dependent mechanism, in order to cause stomatal closure (Zhang et al. 2004). Salt and osmotic stress has been shown to increase expression of the *H^+-ATPase* (Gaxiola et al. 2007). H^+-ATPase is a central enzyme in the acid growth theory that postulates activation of the enzyme by auxin resulting in extrusion of H^+, lowering of the extracellular pH, and loosening of the cell wall that permits cell expansion (Kutschera 2006; Lüthen et al. 1990; Schenck et al. 2010). *TIR1* encodes a protein that is a part of $\mathrm{SCF}^{\mathrm{TIR1}}$ ubiquitin-ligase complex involved in degradation of Aux/IAA proteins and serves as an auxin receptor (Dharmasiri et al. 2005; Kepinski and Leyser 2005; Tan et al. 2007). *HVP1* encodes a vacuolar H^+-inorganic pyrophosphatase that has been shown to be strongly induced by application of exogenous synthetic auxin 2,4- dichlorophenoxyacetic acid (2,4-D) (Fukuda and Tanaka 2006). *YUC1, VT2*, and *NIT2* were chosen as genes representing different auxin biosynthesis pathways. *YUCCA* genes encode a family of flavin monooxygenases that are rate-limiting enzymes in the auxin biosynthesis pathway through tryptamine in *A. thaliana* (Cheng et al. 2006). In addition, expression of *YUCCA* genes has been correlated with endogenous auxin levels in *A. thaliana* and barley (Sakata et al. 2010). *VT2* gene in maize is an orthologue of *A. thaliana* genes belonging to *TAA* gene family. *ZmVT2* was shown to be involved in auxin biosynthesis through indole-3-pyruvic acid (IPA) pathway (Phillips et al. 2011). *NIT2* gene has been implicated in conversion of indole-3-acetonitrile (IAN) to IAA in the indole-3-acetaldoxime (IAOx) auxin biosynthesis pathway in maize (Kriechbaumer et al. 2007).

The expression of *SGT1, ABP1*, and *H^+-ATPase* genes was not changed in *nec1*; however, *TIR1, HVP1, VT2*, and *YUC1* genes were significantly repressed (Fig. 20.3) suggesting that some of the auxin responses are altered in *nec1* mutant possibly due to the altered endogenous auxin level caused by changes in auxin biosynthesis.

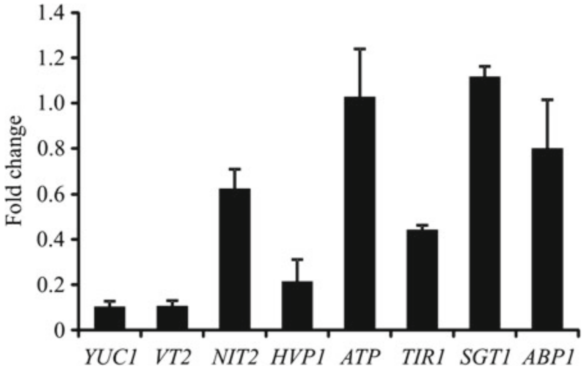

Fig. 20.3 *nec1* mutation affects expression of auxin-related genes in barley. Fold change of transcript abundance in *nec1* relative to wt plants. Error bars represent the standard deviation of means

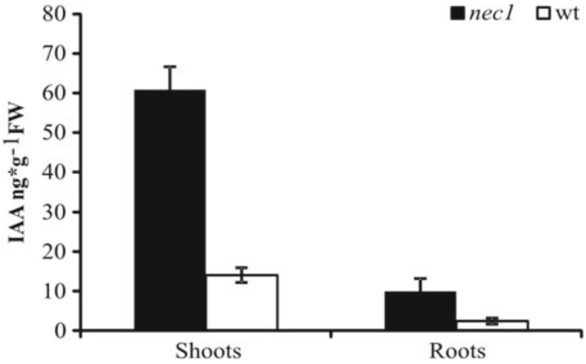

Fig. 20.4 *nec1* exhibits significantly increased endogenous IAA level as quantified by HPLC. Error bars represent the standard deviation of means

20.3.3 IAA Concentration in nec1 Is Significantly Increased

Since significant changes in auxin biosynthesis gene expression were identified in *nec1* mutant, we tested if there are any changes in phytohormone concentration in the *nec1* mutant; therefore, we developed a high-performance liquid chromatography (HPLC) method to simultaneously study the auxin and abscisic acid content in barley leaves and roots (Nakurte et al. unpublished). Highly significant increase of the auxin was observed in both shoots and roots of the *nec1* mutant, while the shoot to root ratio was similar in mutant and wt plants (Fig. 20.4).

Similar increase in auxin concentration was observed in allelic mutants of *nec1* – GSHO989 and FN085 – as well as in the orthologous *A. thaliana dnd2* mutant (data not shown), supporting the hypothesis that mutation in the *CNGC4* gene affects auxin concentration.

20.4 Discussion

Although the role of auxin as a plant disease resistance affecting agent has been suspected for almost two decades, molecular mechanisms linking auxin signaling and plant immunity are largely unknown (Kazan and Manners 2009). Antagonistic interaction with SA-mediated signaling is one of the proposed mechanisms explaining the negative effect of auxin on plant disease resistance (Chen et al. 2007; Wang et al. 2007; Iglesias et al. 2011). Interestingly, several SA-overaccumulating mutants are also impaired in auxin signal perception and vice versa – mutations impairing auxin-related signaling also affect disease resistance. For example, *A. thaliana* SA-overaccumulating mutants *cpr6* and *snc1* produce less IAA, display reduced apical dominance, and are less sensitive to exogenous auxin (Wang et al. 2007). Besides, over-expression of auxin-related genes *GH3* and *WRKY31* enhanced disease resistance of rice to *Xanthomonas oryzae* and *Magnaporthe grisea,* respectively (Ding et al. 2008; Zhang et al. 2009a).

CNGC4 has previously been shown to affect SA-mediated disease resistance in barley (Keisa et al. 2011) and *A. thaliana* (Balague et al. 2003; Jurkowski et al. 2004). In this study, we show that SA-overaccumulating barley mutant *nec1* comprising nonfunctional CNGC4 displays physiological responses resembling altered auxin signaling. *nec1* showed altered sensitivity to exogenous auxin as suggested by coleoptile elongation and stomatal conductance. Physiological indicators representing effects of endogenous auxin did not reveal any significant difference between *nec1* and wt plants. *nec1* exhibited somewhat smaller size of epidermal cells and did not show any change in root gravitropic response. Uncertain physiological data prompted us to analyze expression of auxin-elated genes in *nec1*. Expression analysis of auxin-related genes in *nec1* revealed significant repression of *VT2* (*AtTAA1* homologue) and *YUC1* – genes involved in auxin biosynthesis. Although previously, *TAAs* and *YUCCAs* have been suggested to represent two different auxin biosynthesis pathways (IPA and TAM pathway, respectively) (McSteen 2010), recently it has been shown that both *TAAs* and *YUCCAs* are involved in IPA auxin biosynthesis pathway (Won et al. 2011; Mashiguchi et al. 2011). Therefore, significant repression of both *YUC1* and *VT2* genes in *nec1* suggests that IPA pathway of auxin biosynthesis is likely repressed in *nec1* mutant. Low endogenous auxin concentration could explain altered *nec1* sensitivity to low-dose exogenous auxin application and decreased cell size of *nec1*; therefore, we analyzed IAA content of *nec1*. Surprisingly, HPLC analysis identified significantly increased IAA content in *nec1* shoots as well as roots. In view of contradictory results from gene expression and HPLC analyses, further experiments elucidating the exact effect of *nec1* mutation on auxin biosynthesis in plants will be needed. Elevated auxin concentration throughout *nec1* plants suggests that increased sensitivity to auxin is unlikely caused by changes in auxin transport, but rather might result from alterations in auxin biosynthesis or signaling.

Although it still remains to be elucidated how the lack of cyclic nucleotide-gated ion channel 4 in *nec1* leads to increased level of SA and IAA, it might be anticipated that CNGC4 contributes to both pathways through Ca^{2+}/calmodulin regulatory mechanisms.

Although Ca^{2+} permeability of CNGC4 has not been clearly demonstrated (Balague et al. 2003), a related ion channel CNGC2 has been shown to ensure rise of cytosolic Ca^{2+} in response to PAMPs (Ma et al. 2009). Plant CNGCs are negatively regulated by CaM (Kaplan et al. 2007), and HvCNGC4 comprises calmodulin (CaM) binding domain (Rostoks et al. 2006) suggesting that CNGC4 might be involved in Ca^{2+}/CaM-related signaling. Ca^{2+}/CaM have been implicated as secondary messengers in auxin (Yang and Poovaiah 2000), as well as SA signal transduction (Du et al. 2009). Interestingly, another Ca^{2+} permeable channel, calcium exchanger 1 CAX1, has also been shown to affect auxin-related signaling since *cax1* mutants exhibit reduced apical dominance, inhibited lateral root development, and reduced sensitivity to exogenous auxin treatment (Cheng et al. 2003). Moreover, mutations in the barley *HvCAX1* gene provide broad-spectrum resistance to barley stem rust (Zhang et al. 2009b). Taking into account above mentioned evidence, *nec1* may serve as a model system revealing role of SA-auxin-Ca^{2+} crosstalk in plant disease resistance.

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ORIGINAL PAPER

Structures determining bryophyte species richness in a managed forest landscape in boreo-nemoral Europe

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Abstract Forest patches with high biological value are protected as woodland key habitats (WKH), which are identified by the presence of forest structures and indicator species. However, management for conservation needs to consider also managed forests as habitats for species. In this respect, there is a need to set quantitative targets for species and structures at different landscape scales. Due to non-intensive methods of forest management used prior to 1940 in Latvia, it might be expected that large areas of forest have developed structures that can support many species characteristic of natural forests. The aim of the study was to create a model that best described the richness of bryophyte species that are characteristic of natural forests, using forest structures as explanatory factors. The structures and bryophyte communities on living trees and coarse woody debris (CWD) were described in plots along transects blindly placed in areas dominated by State forests under commercial management. Explanatory variables related to tree species composition and tree size explained 54% of the variation in WKH indicator species richness on living trees. The best explanatory factors were maximum diameter of deciduous tree species and CWD. Low richness of total bryophyte and indicator species was found on dead wood, and the amount of variation in bryophyte species richness on CWD explained by explanatory variables was low. The study indicates the importance of deciduous tree substrate in managed forests in maintaining the spatial continuity of epiphytic species diversity. However, the forests in the managed forest landscape did not support high diversity of epixylic species, even in the WKHs, due to low diversity of suitable dead wood substrate.

Keywords Deciduous forests · Dead wood · Indicator species · Woodland key habitat · Nemoral tree species

Abbreviations

AIC	Akaike information criterion
CWD	Coarse woody debris
DBH	Diameter at breast height

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GLM	Generalized linear model
WKH	Woodland key habitat

Introduction

The conservation of the small fragments of old growth forests that remain in boreal Europe is a priority for protection of biological diversity (Söderström 1988b; Hanski 2005; Jonsson et al. 2005; Löbel et al. 2006a). Forest structures, such as old trees, multiple tree species composition, presence of large deciduous trees, multi-aged tree canopies, and large amounts of dead wood of varying sizes and decay stages, indicate long-term absence of human disturbance (Peterken 1996; Kuuluvainen 2002). A large number of species are dependent on these structures (Berg et al. 2002; Nilsson et al. 2002; Pykälä 2007; Hottola and Siitonen 2008), which are missing or in short supply in managed forests (Siitonen et al. 2000; Bobiec 2002; Krankina et al. 2002). Forest stands with high biological value can be identified based on the presence of structures, species and processes that are indicators of minimal human disturbance (Nilsson et al. 2001). Woodland key habitats (WKH), which are small habitat patches with high biological value, are identified by their structures (Timonen et al. 2010) and/or species composition (Ek et al. 2002). An effective tool in biodiversity conservation is to determine quantitative targets at various landscape scales (Angelstam et al. 2004; Ranius and Fahrig 2006; Villard and Jonsson 2009) for species, structures and natural processes. To set these targets, information is needed on the threshold amounts of structures that are necessary to support healthy populations of species in the landscape.

Many bryophyte species depend on specific microhabitats that are not present in younger forests (Söderström 1988b; Rajandu et al. 2009). These species may be lost with a break in spatial and temporal continuity of their habitats (Snäll et al. 2004; Löbel et al. 2006a, b; Fritz et al. 2008). Higher bryophyte diversity has been observed in old forests compared with managed forests (Andersson and Hytteborn 1991; Vellak and Paal 1999). Stand structures associated with specific bryophyte species include large living trees (Snäll et al. 2003; Kouki et al. 2004) and large dimension dead wood in particular decay stages (Söderström 1988a, b). However, the species richness of bryophytes can be high also in commercial forests (Lõhmus et al. 2007) and new forests (Lõhmus and Lõhmus 2008), provided the presence of suitable substrates for colonization (Ohlson et al. 1997). Species such *Isothecium alopecuroides*, *Homalia trichomanoides* and *Lejenea cavifolia* are not limited by poor dispersal, but rather by presence of substrate (Löbel et al. 2006a; Löbel and Rydin 2009). This suggests that the diversity of bryophytes in commercial forests can be improved by using forest management methods that increase the diversity and amounts of structures required as substrate for species typical of undisturbed forest.

In Latvia, where the legacy of commercial forestry and shifting agriculture extends to the entire forest area, the area of forests older than minimal cutting age is extremely small for all tree species (Tērauds et al. 2011). However, due to non-intensive methods of forest management used prior to 1940, it might be expected that many forest stands have developed structures that can support diversity of species characteristic of natural forests (Tērauds et al. 2011). This is also suggested by the higher mean amounts of dead wood (17.7 m^3/ha) in Latvia, being over two times greater than those in Fennoscandia (MCPFE 2011). In forests dominated by nemoral tree species in Latvia, the presence of epiphytic

WKH indicator species was found to be related to the presence of suitable structures in stands, but fragmentation of these forests also had an effect on presence of some species (Ikauniece et al. 2012).

The aim of this study was to determine the relationship between forest structures and richness of bryophyte species characteristic of natural forests (WKH indicator species) in a broad range of forest types. To achieve this, structures and bryophyte communities were described in plots along transects randomly placed in a forest matrix dominated by State forests under commercial management.

Materials and methods

Study area

Latvia is located in the boreo-nemoral vegetation zone, where boreal coniferous forests are mixed with nemoral forests (Sjörs 1963). The mean annual temperature is 5.0–5.2°C (Lizuma et al. 2007) and the mean annual precipitation is 703 mm (Briede and Lizuma 2007). The study was carried out in the northern part of Latvia in the Ziemelvidzeme Biosphere Reserve (Fig. 1). The Reserve has a total area of 474,350 ha (including territory of sea), of which about 221,383 ha are covered by forest. Within the Ziemelvidzeme Biosphere Reserve, four landscapes composed mainly of state-owned forests were chosen, with a total area of about 30,000 ha. State forests were chosen for study due to availability of digital inventory data. The status of a biosphere reserve does not involve a stricter conservation regime. However, within the reserve there are protected territories with logging restrictions. In addition, many stands have been designated as WKH, which have been voluntarily protected within the Forest Stewardship Council (FSC) forests certification scheme. In the selected 4 state forest areas, 10.4% of the area had some form of logging restriction (for example, only selective cutting allowed or all wood removal banned).

In the chosen forest landscapes, about 37% of the area was on dry soils. Of the wet soil area, 27% was on wet mineral soil, 14% on peat, and the remaining 22% had been drained (Tērauds et al. 2011). The forests were dominated by coniferous tree species (*Picea abies* and *Pinus sylvestris*). The most common deciduous tree species were *Betula pendula*, *B. pubescens*, *Alnus glutinosa, Alnus incana,* and *Populus tremula* (Tērauds et al. 2011). The nemoral tree species *Tilia cordata* and *Fraxinus excelsior* were less common, and *Quercus robur* was rare in the area.

In the four study areas, transects with a total length of 20 km were drawn blindly on digital orthophoto maps; the only subjective criteria used in placing transects were avoidance of agricultural and private lots within the areas. Plots with size $50 \times 2\ \mathrm{m}^2$ ($100\ \mathrm{m}^2$) were placed every 100 m along transects, giving a total of 200 plots. Preliminary study had shown that this plot size would provide sufficient amounts of structures in each plot, although the plots clearly were not representative of whole forest stands.

Data collection

In the field, locations of plots along the transects were established using a Global Positioning System. The field work was conducted from August to October in 2009. In each sampling plot, diameter at breast height (DBH) was recorded for each living tree species. The two birch species (*Betula pendula* and *B. pubescens*) were considered together due to

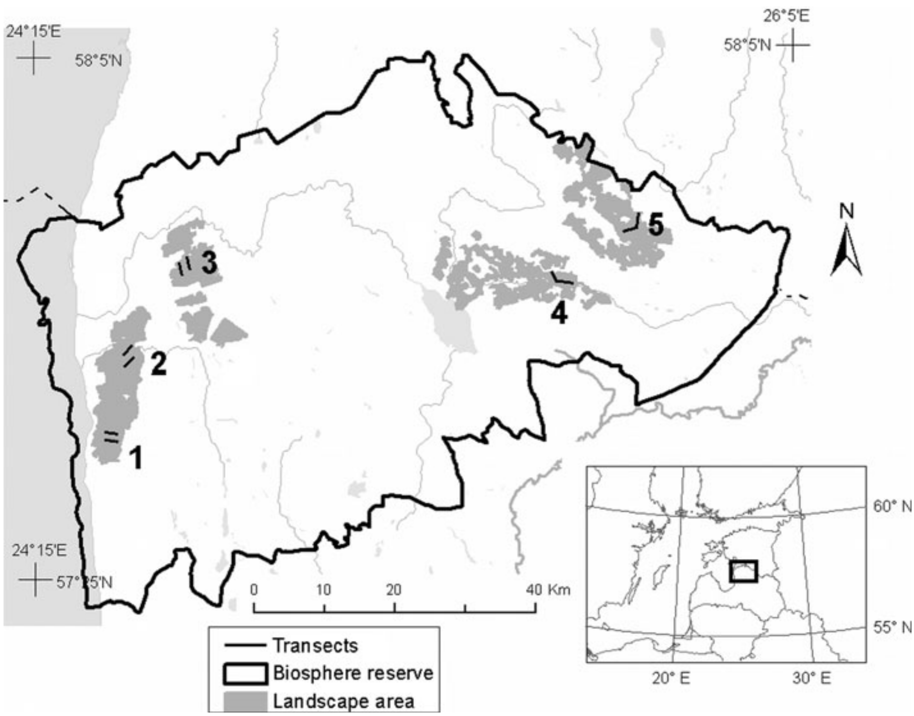

Fig. 1 Location of studied four landscape areas (*shaded*) and five transects (1–5) in the North Vidzeme Biosphere Reserve

similarity in epiphytic communities (Barkman 1958). Stumps were counted by tree species. All dead wood originating inside plots was measured. DBH of snags was measured. For downed trees originating in plots, also when broken into logs, diameter was measured at breast-height length from tree base. Dead branches from trees were not recorded. In such a way, each dead tree was measured only once.

Age of stands was considered as age of the oldest tree layer for the stand in which the plot occurred. This, along with designation of protection status, was taken from inventory data in the Latvian State Forest Register.

In each plot all bryophyte species found on three randomly chosen living trees in the overstorey, three stumps and three downed trees (if present) were recorded. On tree stems, bryophytes were recorded from tree base up to a height of 2 m, which was considered to be a sufficient height to capture most species. Nomenclature followed Hill et al. (2006) for mosses and Grolle and Long (2000) for liverworts.

Data analysis

The effects of substrate variables on bryophyte richness in each plot were determined using a Generalized Linear Model (GLM) with a Poison error distribution and log link function. For living trees the quantitative substrate variables used were: total number of stems, number of stems in diameter classes (≤10.0 cm, 10.1–20.0 cm, 20.1–30.0 cm, 30.1–40.0 cm, >40.0 cm), mean DBH, maximum DBH and basal area (m^2) for each tree species in plots, and grouped by coniferous species (*Pinus sylvestris*, *Picea abies*), nemoral tree species (*Fraxinus excelsior, Quercus robur, Tilia cordata, Ulmus glabra, Acer platanoides*) and other deciduous tree species (*Alnus glutinosa, Betula* spp., *Populus tremula*). The above variables were also calculated for downed trees, snags and total dead wood (downed trees and snags). In addition, the number of stumps, stand age (quantitative variable) and designation as a WKH (bionomial variable) were included as environmental variables.

An iterative process was used to develop models that predict bryophyte and indicator species richness. First, GLM models were tested for each of the above 317 variables. Then, the statistically significant ($P \leq 0.05$) model with the lowest Akaike's information criterion (AIC) was chosen from all possible models with two independent variables (using only variables with P-values ≤ 0.1). This model was used to build a further multi-factor model, with increasing number of independent variables, as long as the new model was significant and a decrease of AIC was found. Models were derived for total bryophyte species richness and WKH indicator species (Ek et al. 2002) on living trees and on coarse woody debris (CWD) plus stumps. The sets of substrate explanatory variables for living trees and dead wood were examined separately, as it was considered that the species pools differ, at least for non-generalist species (Ek et al. 2002). Spearman rank correlation coefficients were calculated to test for significant relationships between total number of all bryophyte species and indicator species in plots. The R programme 2.12.1 version "stats" package was used in the statistical tests.

Results

Of the 200 plots, five were in clearcuts, 38 in WKH and stumps were recorded in 122 plots. The plots greatly varied in tree species composition (Table 1) and age. Of the 200 plots, 65 were in stands with maximum tree-layer age less than 50 years and 79 had age 51–100 years. The remaining plots (56 plots) were located in stands older than 100 years. The most common tree species in the plots were *Picea abies*, *Betula* species (*B. pendula* and *B. pubescens*) and *Alnus glutinosa*. The majority of plots (162 of 200) also contained CWD. Only 16 plots contained CWD that was more than 30 cm in diameter (Fig. 2). *Populus tremula* had the largest mean (32 cm) and maximum DBH (78 cm), but this species was found in only 18 plots (Table 1). For the other tree species the mean and maximum DBH did not exceed 29 and 53 cm, respectively.

Bryophyte species richness

In total, 56 bryophyte species (45 mosses and 11 liverworts) were recorded in the studied sites (Table 2). Of these, 46 species (37 mosses and 9 liverworts) were found on living trees and 41 (33 mosses and 8 liverworts) on CWD (snags and logs) and stumps (Table 2). Eighteen bryophyte species were found only on living trees and 10 species only on decayed wood. Eight bryophyte species were found once in the 200 plots.

Eight (4 mosses and 4 liverworts) WKH indicator species were found in the plots (Table 2). The maximum number of indicator species found in a plot was four, and 30 of the 200 plots contained at least one indicator species. Most of the indicator species were epiphytes (4 mosses and 2 liverworts), the most common being *Neckera pennata*. Two epixylic species (*Nowellia curvifolia* and *Anastrophyllum hellerianum*) were recorded, of which the latter was found only once (Table 2).

Table 1 Summary statistics of living trees and coarse woody debris (CWD) recorded in the 200 plots

Tree composition	Living trees			Coarse woody debris		
	Number of plots	Mean diameter (cm)	Maximum diameter (cm)	Number of plots	Mean diameter (cm)	Maximum diameter (cm)
Pinus sylvestris	52	30	53	26	13	30
Picea abies	139	17	53	120	10	44
Betula spp.	114	18	53	77	11	46
Alnus incana	15	11	23	13	8	18
Populus tremula	18	32	78	11	20	33
Alnus glutinosa	59	20	46	24	13	30
Salix caprea	7	9	17	5	7	13
Fraxinus excelsior	23	16	39	26	9	26
Tilia cordata	22	14	40	9	12	36
Ulmus glabra	7	18	31	2	7	9
Acer platanoides	4	12	22			
Sorbus aucuparia	1	11	11			
Quercus robur				1	26	26
Coniferous trees	153	20	53	128	10	44
Nemoral tree species	45	15	40	36	10	36
Other deciduous species	137	19	78	97	11	46

Number of plots in which a specific substrate was recorded, as well as the mean and maximum values for tree substrate DBH, are given

GLM models for bryophyte species richness

The GLM models explaining the greatest amount of variation in bryophyte richness are shown in Table 3. A total of 40.6% of the variation in total bryophyte species richness on living tress was explained by explanatory variables in the best GLM model obtained. The maximum DBH of deciduous species were the best explanatory variables, with maximum DBH of *Betula* spp. and *Populus tremula* together explaining 27.27% of the total variance (Table 4). Stand age was also a significant variable, but explained a relatively small amount of variation. An even greater amount of variation (54%) was explained in the GLM model for indicator species richness on living trees (Model A; Table 3); the variables with greatest explanatory power were maximum DBH of nemoral tree species (*Ulmus glabra*, *Fraxinus excelsior*, *Tilia cordata* and *Acer platanoides*), maximum DBH of *P. tremula*, and maximum DBH of CWD (Table 4). A similar amount of variation (53%) was explained in Model B (Table 3). In this model, the maximum DBH of each of the four

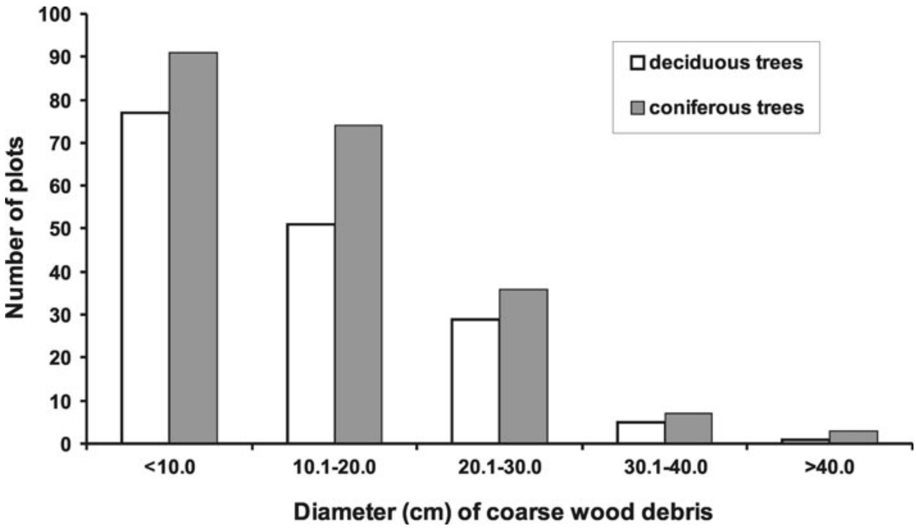

Fig. 2 Number of plots containing coarse woody debris (CWD) in different diameter classes. Diameter was measured at breast-height length from tree base

nemoral tree species in Model A were replaced by the maximum DBH of all of these species as one explanatory variable.

A lower amount of variation of total species richness (16.1%) and indicator species richness (11%) on dead wood was explained by the examined variables. Only basal area of logs and number of stumps in plots were significant variables explaining total bryophyte species richness and only one variable (maximum DBH of CWD) had a significant effect on indicator species richness (Table 3). Designation as WKH was not significantly related to species richness in any of the models.

The total number of all bryophyte species in plots was strongly correlated with the total number of indicator species ($r_s = 0.47$, $P \leq 0.001$). A similar relationship was found between total species and indicator species richness on living trees ($r_s = 0.48$, $P \leq 0.001$). The analysis was not conducted for total number of bryophyte species and indicator species on CWD because of the low number of epixylic bryophyte indicator species recorded.

Discussion

The derived models for bryophyte species richness may indicate direct or indirect relationships with structural features. In the studied forest area, total and indicator species richness of epiphytes on living trees were best explained by the maximum DBH of deciduous tree species. This might suggest the typical species-area relationship (MacArthur and Wilson 1967), as a larger DBH indicates a greater available area for colonization. Tree DBH is a factor that is well know to be related to species richness (Snäll et al. 2003; Snäll et al. 2004; Löbel et al. 2006a, b; Mežaka et al. 2008; Király and Ódor 2010). The association of richness with deciduous trees is also not surprising, considering the greater richness of epiphytes on deciduous trees in comparison with coniferous trees (Löbel et al. 2006b; Mežaka and Znotiņa 2006). Nemoral tree species and *Populus tremula* with DBH >30 cm can be considered as old-growth structures that support biological diversity

Table 2 Number of plots ($n = 200$) in which moss and liverwort species were recorded on different substrates

Species	Number of plots				WKH indicator species
	Substrate			Total number of plots	
	Living trees	CWD	Stumps		
Mosses					
Amblystegium serpens	4			4	
Brachythecium campestre		1	1	2	
Brachythecium rutabulum	40	17	7	57	
Brachythecium salebrosum	13	2		15	
Calliergon cordifolium		4		4	
Calliergonella cuspidata	4			4	
Chiloscyphus pallescens		3		3	
Climacium dendroides	6	2	1	7	
Dicranum montanum	118	15	23	136	
Dicranum polysetum	29	11	12	43	
Dicranum scoparium	71	23	25	96	
Eurhynchium angustirete	51	13	10	60	
Fissidens taxifolius	3			3	
Herzogiella seligeri	4	10	3	16	
Homalia trichomanoides	17	4		19	+
Homalothecium sericeum	5			5	
Hylocomium splendens	34	22	21	61	
Hypnum cupressiforme	51	8	7	61	
Mnium hornum	5			5	
Neckera complanata	1			2	+
Neckera pennata	19	1		20	+
Orthotrichum affine	10			10	
Orthotrichum rupestre		1		1	
Oxyrrhynchium hians	5		1	6	
Plagiomnium affine	8	6	3	16	
Plagiomnium cuspidatum	26	14	4	39	
Plagiothecium curvifolium			1	1	
Plagiothecium laetum	101	4	4	103	
Plagiomnium medium	1			1	
Plagiomnium undulatum	3	3		4	
Platygyrium repens	3	2		5	
Pleurozium schreberi	59	28	31	87	
Polytrichum commune		2	5	7	
Polytrichum juniperinum			2	2	
Ptilium crista-castrensis	3	2	1	5	
Pylaisia polyantha	6			6	
Rhytidiadelphus triquetrus	14	14	8	31	
Rhizomnium punctatum	6			7	
Rhodobryum roseum	3	7	2	11	

Table 2 continued

Species	Number of plots				WKH indicator species
	Substrate			Total number of plots	
	Living trees	CWD	Stumps		
Sanionia uncinata	8	5	3	15	
Sciuro-hypnum oedipodium	1			1	
Tetraphis pellucida	23	5	20	45	
Thuidium delicatulum		3		3	
Thuidium tamariscinum	24		3	15	
Ulota crispa	9			9	+
Liverworts					
Anastrophyllum hellerianum		1		1	+
Blepharostoma trichophyllum	5	2		7	
Frullania dilatata	10			10	
Lejeunea cavifolia	1			1	+
Lepidozia reptans	36	27	10	44	
Lophocolea heterophylla	50		6	72	
Metzgeria furcata	1			1	+
Nowellia curvifolia		12	1	13	+
Plagiochila asplenioides	14	4	1	16	
Ptilidium pulcherrimum	64		9	81	
Radula complanata	35	4		36	

The total number of plots where each species was found, as well as number of plots where found on living trees, coarse woody debris (CWD) and stumps. Woodland key habitat indicator species are indicated

in managed forests (Lõhmus et al. 2005, 2007) in the boreo-nemoral zone. Our results also showed that maximum DBH of *Betula* spp. and *Alnus glutinosa* were explanatory factors for total species richness. This likely represents an indirect relationship, since WKH epiphyte indicator species were lacking on these tree species. The epiphytic bryoflora on *Betula* spp. and *A. glutinosa* species is usually low compared to *P. tremula* and nemoral species such as *Fraxinus excelsior* (Mežaka et al. 2008; Darell and Cronberg 2011), due to the chemical and physical characteristics of bark of these species (Barkman 1958). Thus, *Betula* spp. and *A. glutinosa* with large DBH tended to co-occur in plots that had high bryophyte richness on other tree species.

In a previous investigation on the 70-year forest history in the same area (Tērauds et al. 2011) as the present study, it was suggested that the non-intensive forest methods used prior to 1940 supported regeneration of deciduous species, and that many of these now mature stands have high conservation value. Thus, the observed relationship between maximum diameter of CWD and epiphytic indicator species richness may be explained by minimal human disturbance in the stands with large deciduous trees. The epiphytic WKH indicator species *Homalia trichomanoides* and *Neckera pennata* are limited by habitat quality (Snäll et al. 2004; Löbel and Rydin 2009; Ikauniece et al. 2012), as well as past and present connectivity of habitat (Löbel et al. 2006a). The high frequency of occurrence of *Neckera pennata* (10% of plots) suggests that connectivity of substrate and suitable habitat

Table 3 Summary statistics for building of GLM models

Model	Variance %	Pr (>|z|)	AIC
Living trees			
Total bryophyte richness			
MaxD of *Betula* spp.	18.23	***	1030.2
MaxD *Betula* spp. + MaxD *Populus tremula*	26.2	***	993.1
MaxD *Betula* spp. + MaxD *Populus tremula* + MaxD *Alnus glutinosa*	35.25	***	950.67
MaxD *Betula* spp. + MaxD *Populus tremula* + MaxD *Alnus glutinosa* + MaxD *Fraxinus excelsior*	38.57	***	936.33
MaxD *Betula* spp. + MaxD *Populus tremula* + MaxD *Alnus glutinosa* + MaxD *Fraxinus excelsior* + Stand age	**40.56**	*******	**928.55**
Indicator species richness			
Model A			
MaxD of *Ulmus glabra*	21.75	***	231.61
MaxD *Ulmus glabra* + MaxD *Populus tremula*	35.97	***	204.2
MaxD *Ulmus glabra* + MaxD *Populus tremula* + MaxD *Fraxinus excelsior*	43.66	***	190.28
MaxD *Ulmus glabra* + MaxD *Populus tremula* + MaxD *Fraxinus excelsior* + MaxD CWD	48.34	***	182.59
MaxD *Ulmus glabra* + MaxD *Populus tremula* + MaxD *Fraxinus excelsior* + MaxD CWD + MaxD *Tilia cordata*	51.26	***	178.57
MaxD *Ulmus glabra* + MaxD *Populus tremula* + MaxD *Fraxinus excelsior* + MaxD CWD + MaxD *Tilia cordata* + MaxD *Acer platanoides*	**53.95**	*******	**175**
Model B			
MaxD of broad leaved trees	35.93	***	202.27
MaxD of broad leaved trees + MaxD *Populus tremula*	50.53	***	174.08
MaxD of broad leaved trees + MaxD *Populus tremula* + MaxD CWD	**52.72**	*******	**171.53**
Dead wood			
Total bryophyte richness			
Basal area of downed wood (m^2)	13.43	***	992.36
Basal area of downed wood (m^2) + number of stumps	**16.1**	*******	**975.76**
Indicator species richness			
MaxD of CWD	**11**	******	

Models are produced for total and indicator bryophyte richness on living trees and on CWD plus stumps. For each GLM model, the amount of variance explained, significance level and AIC are shown. The best models are noted in *bold*. Two models are shown for indicator species richness on living trees. *MaxD* maximum DBH

*** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$

are not limiting in the study area. However, several bryophytes listed as WKH indicator species (e.g. *Anomodon* spp., *Isothecium alopecuroides,* and *Antitrichia curtipendula*) were not recorded in the study. The absence of these species is likely due to an insufficient time period since human disturbance to develop old and large diameter trees (Löbel et al. 2006a; Löbel and Rydin 2009).

Basal area of CWD and number of stumps were found to be the best variables explaining total bryophyte species richness on dead wood and the only significant variable

Table 4 Summary statistics for explanatory variables in GLM models with lowest AIC

Variable	Coefficient	Variance %	Pr (>|z|)
Living trees			
Total bryophyte richness			
MaxD *Betula* spp. + MaxD *Populus tremula* + MaxD *Alnus glutinosa* + MaxD *Fraxinus excelsior* + Stand age			
MaxD of *Betula* spp.	0.0171	18.2	***
MaxD of *Populus tremula*	0.0147	9	***
MaxD of *Alnus glutinosa*	0.0127	7.9	***
MaxD of *Fraxinus excelsior*	0.0173	3.3	***
Stand age	0.0025	1.9	**
Indicator species richness			
Model A			
MaxD *Ulmus glabra* + MaxD *Populus tremula* + MaxD *Fraxinus excelsior* + MaxD CWD + MaxD *Tilia cordata* + MaxD *Acer platanoides*			
MaxD of *Ulmus glabra*	0.0937	21.7	***
MaxD of *Populus tremula*	0.0300	14.2	***
MaxD of *Fraxinus excelsior*	0.0481	7.2	***
MaxD of CWD	0.0455	4.7	**
MaxD of *Tilia cordata*	0.0299	3.9	*
MaxD of *Acer platanoides*	0.0743	3.3	**
Model B			
MaxD of broad leaved trees + MaxD *Populus tremula* + MaxD CWD			
MaxD of broad leaved trees	0.1022	35.9	***
MaxD of *Populus tremula*	0.0346	14.6	***
MaxD of CWD	0.0321	2.1	*
Dead wood			
Total bryophyte richness			
Basal area of downed wood (m^2) + number of stumps			
Basal areaof downed wood (m^2)	8.1631	13.4	***
Number of stumps	0.0392	2.6	***
Indicator species richness			
MaxD of CWD			
MaxD of CWD	0.0646	11	**

Standardized coefficients, variance explained and significance levels of explanatory variables are shown. Proportion of variance explained by variables in the GLM models was calculated in an Anova test. *MaxD* maximum DBH

*** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$

explaining indicator species richness on this substrate was maximum diameter of CWD. The relationship between log diameter and frequency of epixylic bryophytes has been observed previously (Andersson and Hytteborn 1991; Kruys et al. 1999; Ódor and van Hees 2004). However, in the present study the amount of variance explained in the models for epixylic species was low (16.1 and 11.0%, respectively). Also, the number of bryophyte species observed on decayed wood was low, compared with species numbers reported in other studies (Lõhmus and Lõhmus 2008; Vellak and Paal 1999), particularly regarding liverworts. Older forest stands tend to contain more bryophyte species, especially threatened and red listed species (Löbel et al. 2006a; Fritz et al. 2008; Rajandu et al. 2009). Many rare bryophytes, such as *Lophozia incisa, Jungermannia leiantha* and *Riccardia latifrons*, can be found in old managed forests (Perhans et al. 2007), but these species were not found in our study. *Anastrophyllum hellerianum*, which was found only once in the present study, requires large diameter logs as habitat (Āboliņa 2008) and is found mostly in old growth forests (Andersson and Hytteborn 1991).

The poor relationship derived for bryophyte richness on dead wood in the present study may be due to a small plot size, as the amounts, size and variability in decay stages of the dead wood sampled in plots may not be representative of that found in stands. However, there was no lack of dead wood in the plots, and the 200 plots can be considered sufficient to represent the dead wood amounts and bryophyte richness on a landscape level. Dead wood was found in 81% of plots, but there was a low supply of dead wood in large diameter classes (>30 cm), which likely is the reason for the observed low richness of epixylic bryophytes. The high frequency of occurrence of dead wood in the plots (Fig. 2) indicates minimal human disturbance in the studied state-forest areas, as suggested previously (Tērauds et al. 2011). However, this period has not been long enough to build the whole spectrum of structures required for specialist species typical of natural forests, particularly regarding CWD with DBH larger than 30 cm. The low frequency of occurrence of plots with large-diameter dead wood can be explained by a history of wood removal, as cut stumps were found in 61% of plots.

Total and indicator species richness were not related to designation of sampled stand as a WKH. This suggests that not all of the plots supporting WKH indicator species have been identified as WKH. Perhaps, these stands may have been too young, or lacked sufficient amounts of structural elements to meet WKH criteria. It is known that many WKH indicator species, such as *Neckera pennata*, *Homalia trichomanoides* and *Lejenea cavifolia* can quickly colonize large-diameter aspen and other suitable substrate also in managed forests, as previously observed in Estonia (Vellak and Paal 1999). Thus, deciduous tree substrate in managed forests is important in maintaining the spatial continuity of epiphytic species diversity. Even a single deciduous tree in coniferous forest creates a suitable habitat patch for many bryophyte species (Löbel et al. 2006a). In the present study, the forests in the managed forest landscape did not support high diversity of epixylic species, even in the WKHs, due to low diversity of suitable dead wood substrate. However, we can expect that substrate diversity and richness of indicator bryophytes will increase in the stands protected as WKHs. In the commercial forests in the area, silvicultural methods should be aimed on maintaining nemoral tree species and *Populus tremula* in the landscape. This can be achieved by retaining these species when present in natural regeneration after logging, and by focus on these species as green retention trees.

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Changes in climatic signals of English oak tree-ring width and cross-section area of earlywood vessels in Latvia during the period 1900–2009

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ABSTRACT

We investigated changes in response of wood formation in English oak (*Quercus robur* L.) to climatic factors since 1900. It was hypothesised that the effect of winter and spring temperatures has weakened, while summer precipitation has become limiting. Increment cores were taken from 40 sites across Latvia. Tree-ring width and cross-section area of earlywood vessels were measured and cross-dated. Regional chronologies were built by pooling time series of trees within two regions of Latvia (western and eastern region), which differed in continentality. Climatic signals differed between the proxies (tree-ring width and earlywood vessel cross-section area) and between regions. Mean cross-section area of earlywood vessels was mainly influenced by temperature during winter and spring. However, the temperature-sensitivity of this proxy to spring to winter temperature weakened towards the end of the previous century, while a positive effect of August precipitation and negative effect of July temperature in the previous year on tree ring width appeared. Thus, climate change has led to lower sensitivity of wood formation of oak to winter temperature and growing season length. However, growth can be limited by summer droughts, which are becoming more common, particularly in the eastern region of Latvia.

1. Introduction

Climate changes are causing shifts in distribution ranges of ecosystems and species (Harrison et al., 2006; IPCC, 2007; Kullman, 2008; Sykes and Prentice, 1996; Walther et al., 2002). These changes, which are most evident on distribution borders where the effects of limiting factors have weakened (Briffa et al., 1998; D'Arrigo et al., 2008; Jacoby and D'Arrigo, 1995; Vaganov et al., 1999), can result in the phenomenon called the "divergence problem" (D'Arrigo et al., 2008). On the northern distribution limit of trees at the tree-line, temperature is becoming a less significant factor for tree-ting width (Briffa et al., 1998; Lloyd and Fastie, 2002). Knowledge of the changes in effect of limiting factors on tree growth is needed to predict changes in tree species distribution and forest dynamics (Fritts, 2001; Iverson and Prasad, 1998). Each species has specific plasticity in response to the environment, which needs to be taken into account when ecological models are constructed (Frank and Esper, 2005; Iverson and Prasad, 1998; Sykes et al., 1996).

Global warming has been most rapid since the 1970s (IPCC, 2007). In Northern Europe autumn and spring temperatures have risen by 0.9 °C, extending the growing season by ~10 days (Kalvāne et al., 2009; Linderholm, 2006; Menzel et al., 2006; Sparks and Menzel, 2002), while precipitation has slightly increased and become more variable (Benestad, 2005; Klavins and Rodinov, 2010; Morberg and Jones, 2005). In Latvia, the highest significant increase of temperature during the last 150 years has occurred in spring, autumn and winter (2.3, 2.1 and 1.8 °C, respectively) (Lizuma et al., 2007). Yearly precipitation has significantly increased during the last 50 years, due to increased amounts during the cold period of the year (Briede and Lizuma, 2007). Regarding extreme events, there has been a significant decrease in frequency of days with very low temperatures in winter and significant increases in the frequencies of very hot days and days with heavy precipitation in summer (Avotniece et al., 2010). Low precipitation and drought periods have been observed to be associated with decreased growth of oak in many regions (Cedro, 2007; Friedrichs et al., 2008; García-González and Eckstein, 2003; García-Suárez et al., 2009; Helama et al., 2009; Jones, 1959; Kelly et al., 2002; Läänelaid et al., 2008; Rozas, 2005; van der Werf et al., 2007; van Hees, 1997), including in Latvia (Matisons and Brūmelis, 2012). However considering that temperature in the dormant period affects growth of English oak (*Quercus robur* L.) close to its northern distribution limit (Drobyshev et al., 2008; Helama et al., 2009; Läänelaid et al., 2008; Matisons and Brūmelis, 2012), increased growth might be expected due to rise of temperature, as reported for other species at high northern latitudes (Briffa et al., 1998; Lloyd and Fastie, 2002).

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Tree-ring width has been widely used to investigate relationships of wood formation and environmental factors (Drobyshev et al., 2008; D'Arrigo et al., 2008; Friedrichs et al., 2008; Fritts, 2001; Kelly et al., 2002; Rozas, 2005; Vaganov et al., 1999), but this proxy can be associated with high autocorrelation and it represents the integrated response to many environmental factors, which may interfere with the climatic signal (Fritts, 2001; Speer, 2010). In ring-porous pedunculate oak earlywood vessels (EVs) are the main water conducting elements (Carlquist, 2001; Tyree and Ewers, 1991). EV are formed in spring and embolize during winter (Cochard and Tyree, 1990; Copini et al., 2010; Hacke and Sauter, 1996; Sperry and Sullivan, 1992; Tyree and Cochard, 1996). Parameters (size) of EV have been described as specifically related to climatic factors before and during their formation (Fonti and García-González, 2008; Fonti et al., 2010, 2007; García-González and Eckstein, 2003; Woodcock, 1989) and EV size has also been linked with tree vigour (Fonti et al., 2009). Compared to tree-ring width, EV series contain less autocorrelation (Fonti and García-González, 2008; Matisons and Brūmelis, 2012; Tardif and Conciatori, 2006).

The aim of the study was to determine changes in response of high frequency variation of tree rings and EV size of English oak to climatic factors during the past 100 years in Latvia. We might expect that the effects of limiting factors on tree-ring width and other wood anatomical structures have already changed for English oak in Latvia, which is located close to its northern limit. We hypothesised that the effect of winter and spring temperature as a limiting factor has weakened, while summer precipitation has become limiting. As the climate in Latvia changes from maritime to continental in the eastern direction, western and eastern regions were considered separately.

2. Material and methods

2.1. Study area

The study was carried out in Latvia, which is situated in the Eastern European plain in the temperate climatic zone (Pūriņš, 1975). Air masses from the Atlantic Ocean and Baltic Sea create a relatively mild climate in Latvia (Klavins and Rodinov, 2010). According to data from the Latvian Environment, Geology and Meteorology Centre (LEGMC), mean monthly temperatures range from −3 to −7 °C in January and from 16 to 17.5 °C in July. Absolute recorded extremes are −43.2 °C (1956) and +36.4 °C (1943) in February and August, respectively. The mean annual temperature is about +5 °C. Mean precipitation in different areas of Latvia ranges from 550 to 850 mm annually. The distribution of precipitation is determined by physiogeographical features: precipitation is the highest on uplands and west-facing coastal areas. More precipitation falls in June–August. Precipitation usually exceeds evapotranspiration. The vegetation period extends from mid April to mid October. Continentality increases in the East direction with increasing distance from the sea (Klavins and Rodinov, 2010). The climate in western Latvia is milder, with lower yearly amplitude of temperature, and more precipitation falls in the eastern region (Table 1). Differences in temperature between the regions are greater in summer and winter. Mean July temperatures are +15.2 and +16.9 °C, and February temperatures are −3.6 and −6.4 °C in the western and eastern regions, respectively. The precipitation sum in July varies from 67.4 mm in the western region to 86.7 mm in the eastern region.

English oak in Latvia is situated close to its northern distribution limit, which on the eastern side of the Baltic Sea extends into Estonia and Southern Finland (Jones, 1959; Hytteborn et al., 2005). Oak-dominated stands cover only 0.3% of the total forest area and mean stand size is ~1 ha (Latvia State Forest Service). In Latvia, which is located in the boreo-nemoral zone (Sjors, 1963), oak often is mixed with coniferous tree species (Latvian Forest Service).

Table 1
Mean monthly temperature and precipitation in western and eastern regions (Fig. 1) for the period 1900–2000. Values were calculated from data obtained from the Liepāja (western region) and Daugavpils (eastern region) meteorological stations.

	Region	
	Western	Eastern
Temperature (°C)		
October	6.4	5.3
November	2.0	0.0
December	−1.3	−4.2
January	−3.3	−6.5
February	−3.6	−6.4
March	−0.9	−2.5
April	4.2	4.6
May	9.3	11.1
June	12.8	14.9
July	15.2	16.9
August	14.4	15.7
September	10.6	10.6
Precipitation sum (mm)		
October	60.3	61.0
November	52.6	52.2
December	42.3	43.1
January	36.0	36.7
February	27.1	31.0
March	27.6	31.8
April	31.6	37.8
May	39.8	56.2
June	51.7	73.6
July	67.4	86.7
August	65.9	80.1
September	60.5	66.7

2.2. Sampling

Oak stands with age more than 100 years and area more than 1 ha were chosen from the Latvian Forest Service database. Soils in most of the sites were dry and clayey; however, some plots had moist soils. Land relief was mostly flat, or in some cases with slight to moderate slopes. Most of the stands were mixed with conifer and deciduous species (Table 2). Living canopy oaks were cored in 40 sites that were located throughout the territory of Latvia (Fig. 1). In each site it was planned to core 10 oaks from opposite sides of stem at 1.4 m height, but in several sites the number of cored trees differed due lack of suitable trees or trees with wood rot. Cores from some other studies were also used. In these cases the number of trees cored was higher (sampling plots BZN, DOB1, MZN, and RDA), but only one core per tree had been taken. More detailed information about stands is given in Table 2.

2.3. Proxy measurement

In the laboratory cores were dried and glued into fixation planks. Dry cores were gradually sanded with sand paper (grain size 120, 240, 320 and 500) using a vibration sander (Makita BO3700). Sawdust was removed by compressed air. Cores were then treated (rubbed) with chalk to enhance contrast between earlywood and latewood. Core images for further measurements were obtained using an EPSON GT 15,000 scanner with 1200 dpi resolution. Tree-ring width (TRW) was measured using the LignoVision v1.36. program (RinnTECH); tree-ring borders were set manually. For measurement of earlywood vessel cross-section area core images were cut into earlywood images that contained earlywood vessels of the first two or three rows (García-González and Fonti, 2006) in each year tree ring (from 1899). Mean earlywood vessel cross-section area (EVA) was measured from earlywood images

Table 2
Information on stands: geographic coordinates, habitat moisture, relief, estimated mean stand age and stand composition of sampling plots and number of cored trees.

Code	Coordinates	Soil moisture	Relief	Stand age	Mixed stands	Number of cored trees
Western region						
ANC	21°56′5E, 57°34′23N	Dry	Flat	215	Yes	10
CCE	22°34′18E, 56°39′1N	Dry	Flat	176	Yes	10
DOB	23°17′24E, 56°36′9N	Dry	Slight slope	184	Yes	10
DOB1	23°13′10E, 56°35′34N	Dry	Slight slope	192	Yes	18
DRB	21°17′42E, 56°35′0N	Dry	Flat	212	Yes	10
DUN1	22°20′52E, 57°29′47N	Dry	Flat	157	No	10
DZC	23°2′17E, 57°7′5N	Dry	Flat	227	Yes	10
GVZ	21°19′5E, 56°31′26N	Moist	Flat	230	Yes	10
ICV	24°8′56E, 56°34′9N	Dry	Flat	148	No	10
JBRsa	23°23′57E, 56°43′0N	Dry	Flat	237	No	8
JBRsl	23°25′22E, 56°44′51N	Moist	Flat	176	Yes	7
JEL	23°45′0E, 56°37′4N	Dry	Flat	225	Yes	18
KUL	22°2′6E, 56°55′26N	Dry	Flat	118	Yes	11
MZN	24°1′58E, 56°26′47N	Dry	Flat	223	Yes	10
PIL	21°41′53E, 57°12′5N	Dry	Flat	197	Yes	12
SKR	21°59′7E, 56°34′58N	Dry	Slight slope	173	Yes	10
TBR	21°34′23E, 56°45′32N	Dry	Flat	167	Yes	12
UGL	21°58′31E, 57°14′35N	Dry	Flat	223	Yes	14
Eastern region						
AGL	26°54′5E, 56°12′41N	Dry	Flat	206	No	10
ALK	26°57′37E, 57°22′54N	Dry	Slight slope	115	Yes	10
BAR	26°38′59E, 56°42′30N	Moist	Flat	165	Yes	12
BIK	26°17′51E, 56°55′19N	Dry	Flat	160	Yes	10
BZN	26°3′41E, 56°50′11N	Dry	Flat	118	Yes	23
CES	25°13′24E, 57°17′30N	Dry	Slight slope	252	Yes	10
DKL	25°4′35E, 57°37′28N	Dry	Flat	186	Yes	10
ELK	25°36′45E, 56°13′19N	Dry	Flat	118	Yes	11
EZR	27°36′28E, 56°11′15N	Dry	Flat	202	No	10
JEK	25°57′16E, 56°28′17N	Dry	Slope	180	Yes	10
LMBsa	24°55′26E, 57°30′52N	Dry	Flat	184	Yes	10
LMBsl	25°2′11E, 57°30′33N	Moist	Flat	220	Yes	10
LOB	25°12′42E, 56°44′12N	Dry	Flat	193	Yes	16
LZA	27°57′22E, 56°30′23N	Dry	Flat	167	Yes	5
RDA	26°10′57E, 55°53′11N	Dry	Slight slope	180	Yes	14
RUJ	25°23′40E, 57°52′26N	Moist	Flat	114	No	10
SIG	24°48′2E, 57°9′5N	Dry	Slope	219	Yes	12
SKV	25°2′33E, 56°39′38N	Dry	Flat	117	No	10
STP	24°56′45E, 57°22′21N	Dry	Slight slope	180	No	10
STR	25°43′19E, 57°37′44N	Dry	Slight slope	176	Yes	10
VDL	25°46′31E, 56°16′21N	Dry	Flat	173	Yes	10
VLK	26°4′18E, 57°42′21N	Dry	Slight slope	149	Yes	7

using the WinCELL 2007a program with greyscale pixel classification (threshold values from 185–230) and a data filter (cross-section area from 6,500–120,000 μm^2). A batch function was used to measure EVA for each core. Before launching the batch function, 30–40 earlywood images from each of 20 cores were measured manually to ensure measurement accuracy and to adjust measurement parameters if necessary.

2.4. Data analysis

Previously, principal component analysis of site chronologies indicated that high Interseries correlations of site time series of TRW within regions were similar; however they were significantly higher (p-value < 0.05) within each region than between regions, indicating differences of wood formation between regions. Regarding EVA, interseries correlations were significantly higher in the eastern region than in both regions and significantly lower in the western region. The western and eastern regions (Fig. 1) contained 18 and 22 sites, respectively. In each region most of sites were located on dry soil and proportion of sites on moist soils within each region was similar (~10%). Quality of TRW and EVA measurements was verified and cross-dating was made by graphical examination and using the program COFECHA (Grissino-Mayer, 2001). Cross-dating was conducted between cores in each stand, between all cored trees, and between stands (mean time series). As tree-ring series can have high autocorrelation and contain trends that are not related with climatic factors (Fritts, 2001; Speer, 2010), residual chronologies of TRW and EVA were produced based on cross-dated time series of trees for the two regions with ARSTAN (Cook and Holmes, 1996) using double detrending: negative exponential curve and autoregressive modelling. Interseries correlation, autocorrelation, mean sensitivity and EPS (Wigley et al., 1984) were estimated for the TRW and EVA chronologies produced for the regions.

To determine temporal changes in limiting climatic factors, Pearson correlation analysis and response function analysis between regional residual chronologies of TRW and EVA and climatic variables were conducted for the whole period of 1900–2009 and for 50-year moving intervals within that period. The correlation and response function analyses were conducted using DENDROCLIM2002; significance of the coefficients was determined by the bootstrap method (Biondi and Waikul, 2004). Climatic data was obtained from the Latvian Environment, Geology and Meteorology Centre (LEGMC) for the Riga Meteorological Station, as it had the longest record series in Latvia. Monthly climatic data used to test relationships with TRW and EVA extended from April in the year prior to tree-ring formation (p.t.f.) to October of the year of tree-ring formation, for the period 1900–2009.

3. Results

In total, data from 416 trees, which passed quality checking and were crossdated successfully, were used for construction of regional

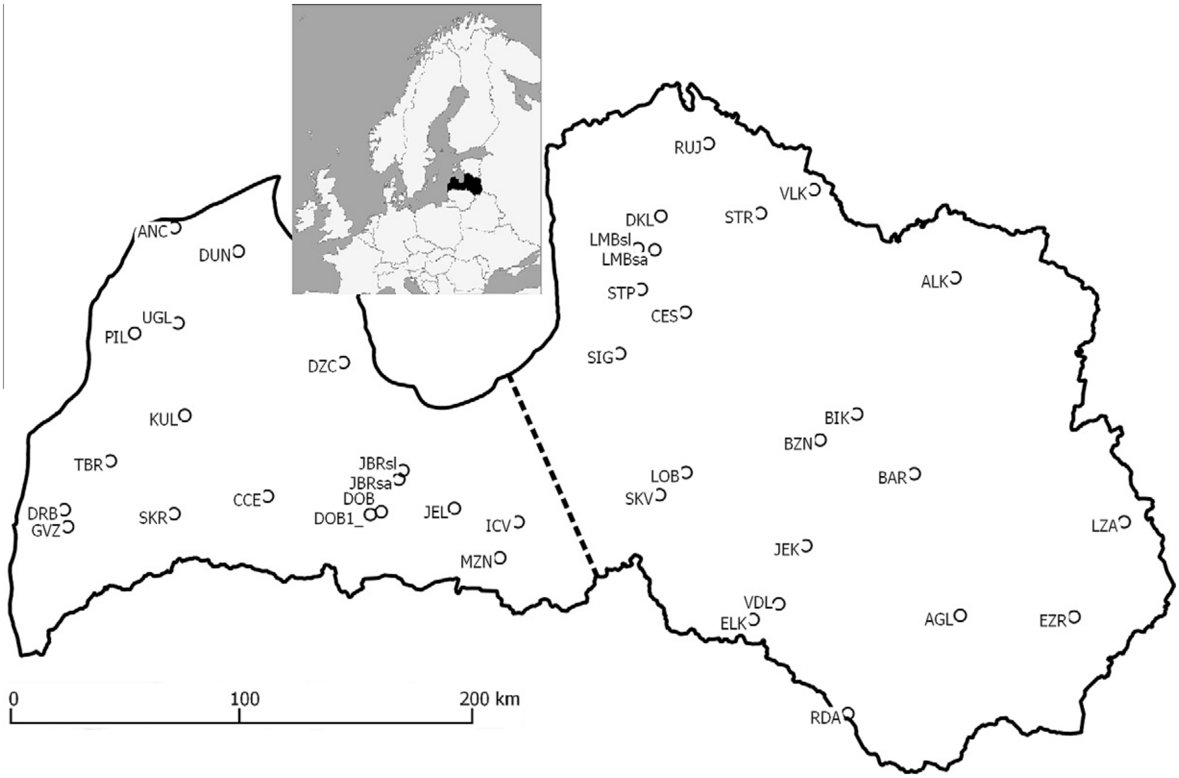

Fig. 1. Location of sampling plots.

chronologies. During crossdating and quality checking more time series of EVA were excluded from analysis compared to TRW, which resulted in lower replication. This was done because EVA showed lower agreement between trees within sites and region. Nevertheless, >70% of the tree time series were crossdated successfully. Statistics for time series are shown in Table 3. TRW showed higher agreement between trees in sampling plots compared to EVA (mean IC = 0.63 and 0.38, respectively), but agreement between mean time series for sites was lower for TRW than for EVA (mean IC = 0.55 and 0.67, respectively). Interseries correlations of site time series of TRW within regions were similar; however they were significantly higher (p-value < 0.05) within each region than between regions, indicating differences of wood formation between regions. Regarding EVA, interseries correlations were significantly higher in the eastern region than in both regions and significantly lower in the western region. TRW contained more autocorrelation than EVA (mean AC = 0.70 compared to 0.47, respectively). Time series of mean TRW and EVA for sampling plots in the period 1900–2009 are shown in Fig. 2. Range in amplitude of TRW tended to decrease with tree age, while mean and range of EVA tended to increase. Also, the spread of EVA series between sites increased after about 1990.

Chronologies of crossdated time series (TRW and EVA) of trees within western and eastern regions of Latvia area shown in Fig. 3. TRW showed higher amplitude of index values compared to EVA. The index values of EVA had extremely low values in several years (i.e. 1939, 1956, 1979 and 1987) for both regions. Both TRW and EVA proxies showed higher amplitude of index values in the eastern region of Latvia. A decrease in amplitude after about 1980 is visible for both proxies, which is more evident for the EVA chronologies. Statistics for the chronologies are shown in Table 4. Although 10% and 45% of EPS values for TRW and EVA within stands, respectively, were below 0.85 (not shown), both EPS and GLK were high (>0.96 and >0.53, respectively) for the chronologies within the two regions, indicating that the datasets contained expressed common signals (Table 4). Autocorrelation and sensitivity of the chronologies were higher for TRW compared with EVA.

The effect of climatic factors was more expressed (higher correlation coefficients) for EVA than TRW, while TRW showed higher agreement within regions (Table 4) and higher range of chronologies (Fig. 3). When the entire period of 1900–2009 was considered, of the tested 19 monthly temperature and 19 monthly precipitation parameters, 13 and 6, respectively, were significantly correlated with TRW and/or EVA in one or both regions (Fig. 4). For this period (Fig. 4), response function analysis indicated significant response of TRW and EVA to only 7 temperature and 2 precipitation factors in one or both regions. Regarding TRW, July and August temperature of the year p.t.f. had significant negative and October temperature had positive effect (both correlation and response) in both eastern and western regions (Fig. 4). Current year March and June temperatures had positive correlation only in the western region and July temperature only in the eastern region. In the western region, November precipitation of the year p.t.f. had negative correlation and April precipitation had positive correlation with TRW. In contrast, in the eastern region, precipitation in July of the year prior to ring formation (p.t.f) and in August of the current year showed significant correlation to TRW, and the latter factor also showed significant response.

Compared to TRW, EVA had a greater number of significant correlations with monthly temperature, and the correlation coefficients were higher (Fig. 4). These relationships appeared to be similar between the regions. Winter and spring temperature (December–April) of the current year, as well as May–June and September temperature of the year p.t.f. were significantly correlated with EVA in both regions. Precipitation had weaker correlation than temperature: a significant effect was evident only for January of the year of tree-ring formation in both regions, and also for June of that year in the eastern region. Although correlations were similar between regions, response function analysis indicated

Table 3
Statistics of mean time series of tree-ring width and earlywood vessel area. Autocorrelation (AC) and interseries correlation (IC) were calculated for the period of 1900–2009.

Stand	Tree-ring width					Earlywood vessels				
	Period	Min–max, 10^2 mm	Mean, 10^{-2} mm	AC	IC	Period	Min–max, 100 μm^2	Mean 100 μm^2	AC	IC
AGL	1805–2010	69–385	163	0.59	0.71	1899–2010	217–646	414	0.55	0.60
ALK	1896–2010	104–590	294	0.77	0.57	1899–2010	123–605	319	0.72	0.57
ANC	1697–2009	52–318	145	0.74	0.46	1899–2009	101–411	241	0.31	0.33
BAR	1845–2009	51–399	154	0.72	0.70	1899–2009	89–419	241	0.47	0.55
BIK	1851–2010	69–423	149	0.62	0.74	1899–2010	223–737	452	0.31	0.58
BZN	1892–2009	39–546	192	0.76	0.69	1899–2009	76–544	270	0.60	0.37
CCE	1834–2009	54–237	112	0.75	0.65	1899–2009	111–422	230	0.32	0.30
CES	1759–2010	61–322	152	0.69	0.59	1899–2010	230–643	402	0.52	0.41
DKL	1825–2010	59–334	144	0.60	0.67	1899–2010	143–612	305	0.52	0.47
DOB	1826–2009	35–240	120	0.84	0.60	1899–2009	114–439	244	0.46	0.32
DOB1	1818–2009	53–268	131	0.59	0.58	1899–2009	178–581	363	0.30	0.29
DRB	1798–2009	57–319	152	0.67	0.61	1899–2010	115–439	244	0.43	0.26
DUN1	1852–2008	67–398	173	0.79	0.55	1899–2008	154–561	302	0.40	0.08
DZC	1782–2008	46–217	113	0.63	0.55	1900–2008	198–514	327	0.31	0.31
ELK	1894–2010	82–486	217	0.81	0.69	1899–2010	142–626	325	0.67	0.44
EZR	1809–2010	49–228	109	0.54	0.71	1899–2010	167–582	324	0.53	0.55
GVZ	1780–2009	49–321	148	0.71	0.66	1899–2009	146–454	275	0.27	0.35
ICV	1862–2009	59–458	208	0.80	0.70	1899–2009	119–552	277	0.45	0.31
JBRsa	1773–2009	60–293	126	0.73	0.52	1899–2009	128–384	226	0.37	0.28
JBRsl	1834–2009	64–381	187	0.69	0.73	1899–2009	124–408	251	0.37	0.34
JEK	1831–2010	86–465	234	0.76	0.64	1899–2010	127–606	293	0.73	0.36
JEL	1785–2009	52–372	170	0.73	0.59	1899–2009	123–479	246	0.44	0.35
KUL	1892–2009	43–411	184	0.78	0.76	1899–2009	84–324	194	0.39	0.31
LMBsa	1826–2009	74–359	176	0.63	0.68	1899–2009	150–505	292	0.43	0.47
LMBsl	1710–2009	53–356	160	0.65	0.69	1899–2009	135–480	303	0.28	0.38
LOB	1816–2008	46–281	125	0.63	0.62	1900–2008	142–676	310	0.51	0.34
LZA	1844–2010	79–625	269	0.84	0.35	1899–2010	143–569	279	0.64	0.18
MZN	1777–2009	46–261	130	0.47	0.63	1899–2009	159–620	378	0.39	0.26
PIL	1813–2009	58–241	121	0.68	0.65	1899–2009	77–331	204	0.38	0.25
RDA	1830–2009	25–309	126	0.70	0.61	1899–2009	150–547	335	0.27	0.35
RUJ	1897–2010	41–483	172	0.83	0.70	1903–2010	93–540	249	0.73	0.44
SIG	1792–2010	42–270	117	0.66	0.68	1899–2010	176–563	324	0.59	0.57
SKR	1837–2009	63–262	127	0.68	0.62	1899–2009	144–500	288	0.42	0.39
SKV	1894–2010	71–619	253	0.83	0.74	1903–2010	94–650	309	0.74	0.48
STP	1831–2010	61–321	159	0.71	0.68	1899–2010	112–453	247	0.55	0.34
STR	1835–2010	46–294	126	0.67	0.58	1899–2010	138–494	282	0.55	0.39
TBR	1843–2009	75–292	164	0.64	0.57	1899–2009	141–580	315	0.32	0.21
UGL	1787–2009	54–255	122	0.72	0.63	1899–2009	139–493	293	0.31	0.26
VDL	1838–2010	59–424	195	0.79	0.68	1899–2010	109–476	261	0.64	0.50
VLK	1862–2010	65–456	193	0.68	0.59	1899–2010	66–393	235	0.66	0.53

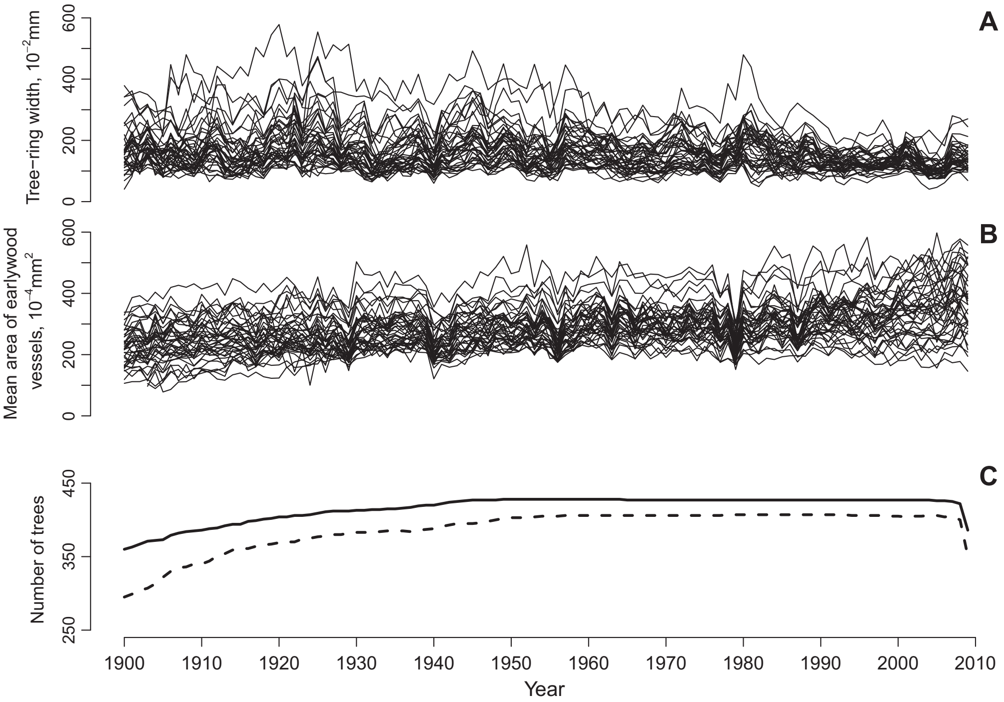

Fig. 2. Mean sampling plot time series of TRW (A), EVA (B) and sampling depth (C, solid line for TRW, broken line for EVA) for the period 1900–2009.

a larger number of significant monthly factors in the eastern region. EVA showed significant response to December and April temperature in both regions and in the eastern region additionally to temperature in September of the year p.t.f., and in January and June (negative) of the current year. EVA also had significant negative response to December precipitation in the eastern region, which was not evident in correlation analysis.

The relationships of TRW, and particularly EVA, with climatic factors (climatic signals) (Figs. 5 and 6) changed during the 20th century. The temperature–EVA relationship for January and February clearly appears to have weakened in the period after the late 1960–1970s, as the correlation coefficients in moving intervals after about 1927–1977 were lower than in previous intervals and response to these factors disappeared at that time. Correlations between March temperature of the current year and October of the year p.t.f. and EVA in moving intervals after 1927–1977 mostly disappeared in later intervals, while the correlation with December temperature increased after this time (Fig. 5). However there was no significant response of EVA to these factors (Fig. 6). In the western region, a significant positive effect of June precipitation of the current year on EVA appeared in moving intervals after 1924–1974, which extended to the interval 1955–2005. In the eastern region, this effect was evident only in mid-intervals of the study period. However, response to June precipitation was significant only in a few intervals in the middle of analysed period in both regions (Fig. 6). In the later part of the investigated period, in the western region the effect of June temperature of the year p.t.f. on EVA strengthened (correlation and response coefficients increased and became significant). In the eastern region, the opposite was observed, i.e. this effect was observed in the beginning of analysed period. A significant negative correlation with August precipitation of the year p.t.f. occurred in some moving 50-year intervals after 1938–1988.

TRW showed less stable correlations with climatic factors, as there was no single climatic factor significant during the whole study period, in contrast to EVA (Fig. 5). There were 17 climatic factors that showed significant correlation with TRW in less than five 50-year moving intervals of the study period. Significant correlation with October temperature of the year p.t.f. (Fig. 5) occurred in moving intervals before 1913–1963 and 1921–1971 in western

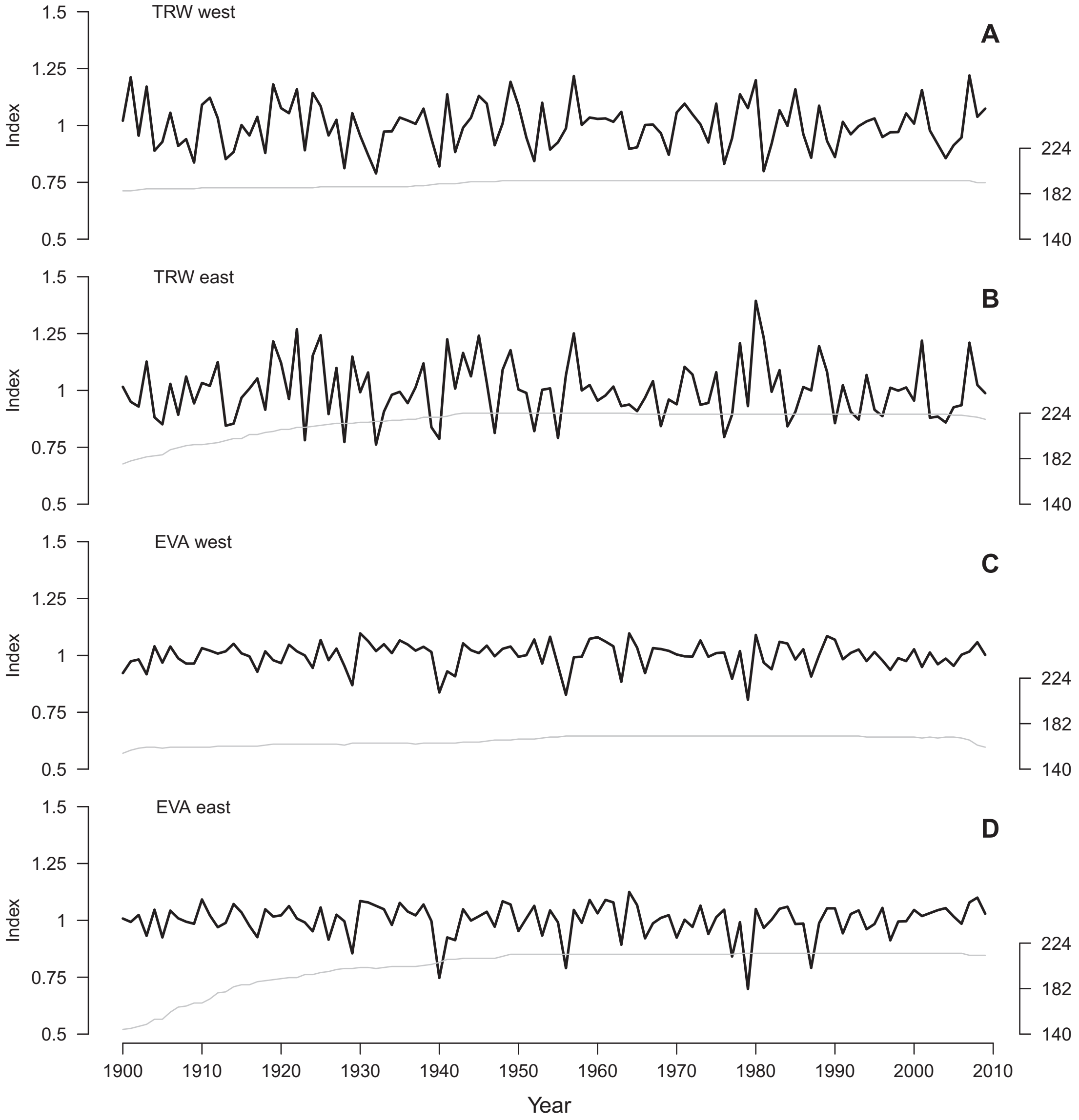

Fig. 3. Residual chronologies of TRW (A western region and B eastern region) EVA (C western region and D eastern region) and sampling depth (number of trees) for the period 1900–2009.

and eastern regions, and this effect was also significant in response function analysis (Fig. 5). In the western region, the effect of current February and March temperature was correlated with TRW only in intervals before 1934–1984 and 1939–1989, respectively. In the eastern region, significant correlation with current February and March temperature also disappeared around that time, but these factors lacked significant correlation in intervals at the beginning of the study period. A negative effect of July temperature of the year p.t.f. was indicated by significant correlation with TRW in most of the intervals of the study period (Fig. 5), but significant response occurred only in seven intervals at the end of the period (Fig. 6). In the eastern region, both correlation and response of current August precipitation became significant in moving intervals after 1929–1979. Precipitation in September of the current year and November and December of the year p.t.f. correlated significantly with TRW only in a few moving intervals, but the former was evident only at the start and the latter two at the end of the study period.

4. Discussion

4.1. Dataset characteristics

Although the number of trees sampled in each sampled stand was low, leading to several low EPS values for stands (Table 3), the agreement between stands was high (mean IC for TRW and EVA was 0.55 and 0.67, respectively), indicating the validity of pooling the data. Validity of dividing plots into the defined regions is supported by higher agreement of time series within than between regions. The EPS values (0.975–0.992) obtained for regions were rather high, indicating that they each contained a common signal (Wigley et al., 1984). However, agreement of series differed between the two regions (Table 4) and range of chronology index values (Fig. 3) was higher in the eastern region, suggesting reduced sensitivity of the tested factors in the more maritime western region.

4.2. Tree-ring width

The significant effect of temperatures in autumn October of the year p.t.f temperature and in March of the current year (Fig. 4) on TRW can be explained by a longer growing period, which presently is about 190 and 175 days in the western and eastern regions, respectively (LEGMC), and by greater accumulation of assimilates (Fritts, 2001; Menzel and Fabian, 1999; White et al., 1999). The amount of stored assimilates can also be important for cold tolerance (Essiamah and Eschrich, 1985; Morin et al., 2007; Repo et al., 2008). The loss of effect of October temperature of the year p.t.f. in moving intervals after the 1960s and a later loss of correlation with March temperature (Figs. 5 and 6) is likely due to increased temperature in these months, and hence also growing season length, as has been observed in Northern Europe (IPCC, 2007; Lizuma et al., 2007). Thus, the present-day growing season length is likely non-limiting for TRW. Loss of correlation with February temperature can be explained by milder winters (Avotniece et al., 2010; Lizuma et al., 2007). However, in the eastern region, where winters are harsher, this effect was observed only in moving intervals in the mid part of the study period, when the increasing temperature trend was slower (Lizuma et al., 2007). The negative correlation of April temperature of the year p.t.f. with TRW in the eastern region during 1934–2000 (Fig. 5) might be explained by shifting weather conditions from warm periods to cold days with snowfall. Wood formation in oak in Latvia begins in April, before bud break (Sass-Klaassen et al., 2011), which in the eastern Baltic region usually occurs in late April to early May (Ahas et al., 2000; Kalvāne et al., 2009). As onset of cambial activity can

Table 4
Statistics of TRW and EVA time series datasets of western and eastern region of Latvia: number of trees, mean interseries correlation, mean sensitivity, mean first order autocorrelation, expressed population signal (EPS), GLK, minimal and maximal size and standard deviation (SD) of TRW (1/100 mm) and EVA (10^{-4} mm^2).

	TRW		EVA	
	West	East	West	East
Number of trees	192	224	167	214
Interseries correlation	0.443	0.481	0.340	0.468
Mean sensitivity	0.218	0.233	0.207	0.192
Autocorrelation (first order)	0.761	0.761	0.373	0.546
EPS	0.975	0.987	0.968	0.992
GLK	0.577	0.59	0.536	0.563
Min	155	167	272	309
Max	912	982	849	891
SD	69	78	64	83

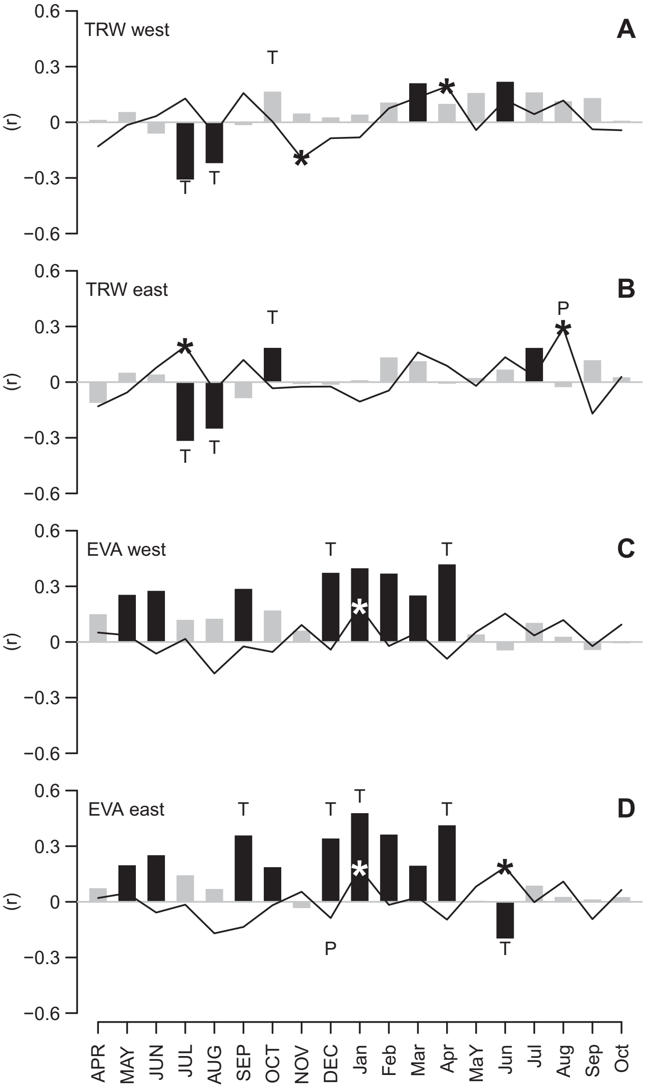

Fig. 4. Pearson correlation coefficients between mean monthly temperature (columns, significant correlations in black), monthly precipitation sums (line, significant correlations marked with *) from April of the year previous to growth to October of year of growth and residual chronologies of TRW (A western region and B eastern region) and EVA (C western region and D eastern region) in the period 1900–2009. In uppercase–climatic factors in the year prior to tree-ring formation. Letters in the plot indicate significant responses to climatic factors, determined by response function analysis (T– temperature, P– precipitation).

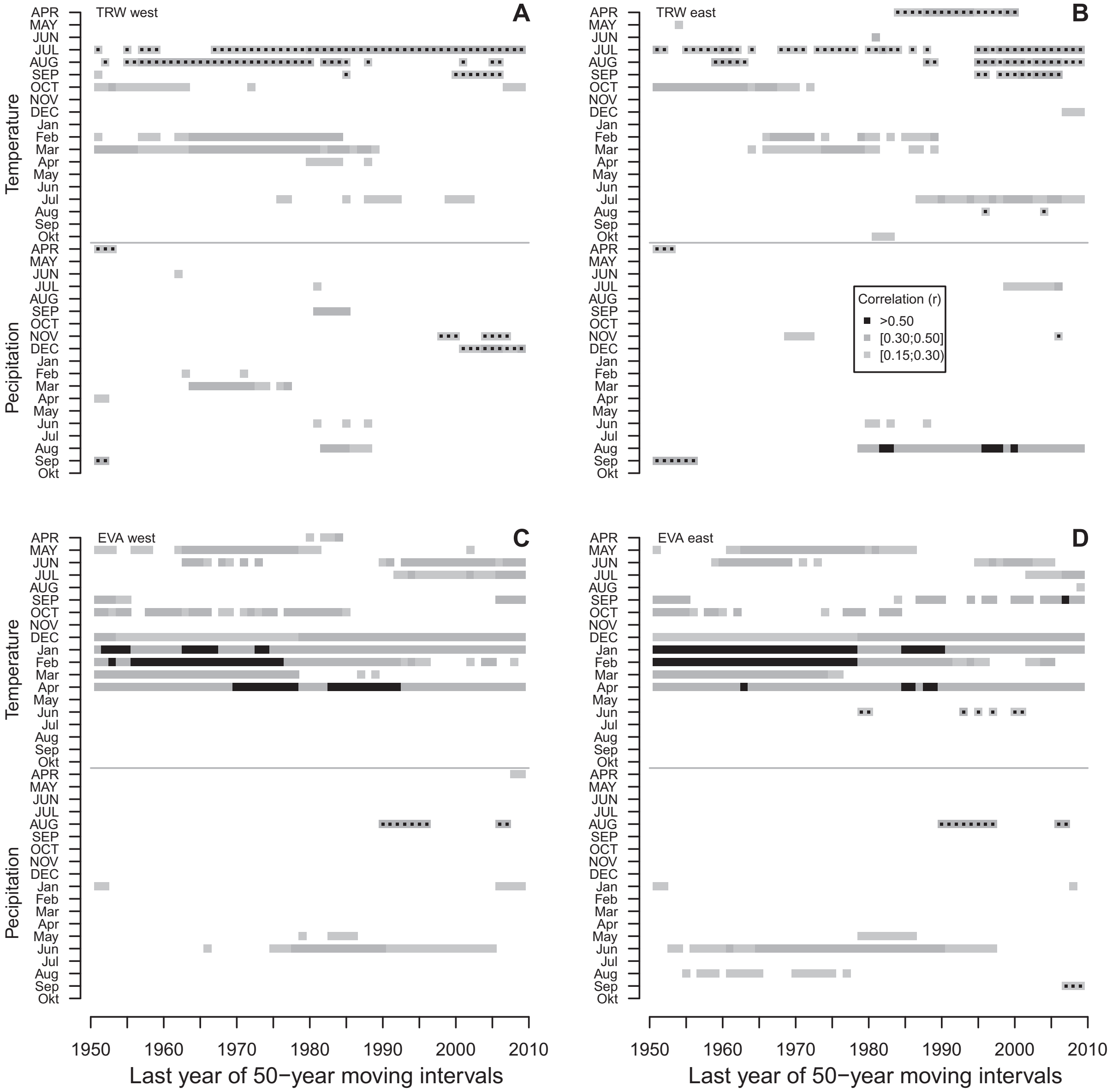

Fig. 5. Significant Pearson correlation coefficients between mean monthly temperature and monthly precipitation sums from April of the year previous to tree-ring formation to October of year of growth and residual chronologies of TRW (A western region and B eastern region) and EVA (C western region and D eastern region) for 50-year moving intervals in the period 1900–2009. Dots represent negative correlation. In uppercase –climatic factors in the year prior to tree-ring formation.

occur earlier in response to higher temperatures (Deslauriers et al., 2008), rapid temperature shifts in spring can cause damage to vessels and meristem and reduce growth (Gu et al., 2008; Zhu et al., 2000).

As suggested by significant correlation with July and August (negative) temperature of the year p.t.f. and current year August precipitation (positive) in the eastern region (Fig. 4), climatic factors related with water deficit in summer appear to be limiting factors for TRW. Correlation and response function analysis (Figs. 4–6) also suggest that this effect of water deficit (p.t.f. July temperature and current year August) has increased. With warming of the climate, precipitation in August now is the main limiting factor for TRW in the eastern region (highest response coefficient values, Fig. 6). Increasing mean July temperature (Lizuma et al., 2007) and increasing number of warm (tropical) nights, when temperature is above 20 °C (Avotniece et al., 2010), might decrease amount of available water and facilitate water deficit in August. In drought conditions (water deficit) assimilation is decreased due to stomata closure and decrease of water potential, resulting in insufficient water supply to leaves (Pallardy and Kozlowsky, 2008). A negative effect of summer temperature is common in central and southern regions of the distribution of oak, which has been explained by occurrence of water deficit (Friedrichs et al., 2008; García-González and Eckstein, 2003; Lebourgeois et al., 2004; Leuschner et al., 2001; Rozas, 2005). The observed negative correlation with summer temperature in the previous year can be explained by autocorrelation. In years with cooler summer temperature (less water deficit) in the year p.t.f., growth in the next year can be influenced via nutrient reserves (Essiamah and Eschrich, 1985; Pallardy and Kozlowsky, 2008; Tardif and Conciatori, 2006), which in oak are accumulated most rapidly around August (Barbaroux and Breda, 2002; Morecroft et al., 2003; Xu and Griffin, 2006).

However, as the sampled trees increased in age during the investigated period, the observed possible drought effect might reflect increased maintenance costs in older trees (Ryan, 1990), leading to an age-related change in sensitivity (Rozas, 2005). Also,

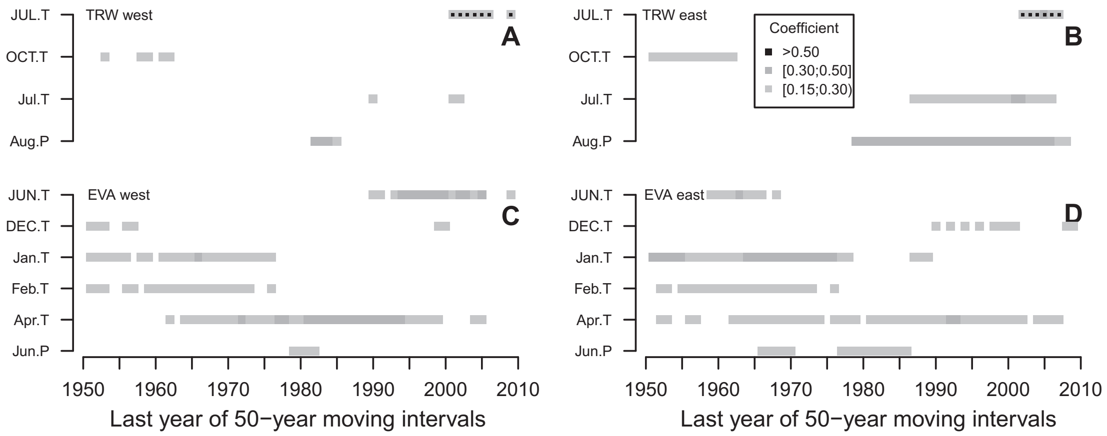

Fig. 6. Significant response function coefficients between mean monthly temperature (T) and monthly precipitation sums (P) from April of the year previous to tree-ring formation to October of year of growth and residual chronologies of TRW (A western region and B eastern region) and EVA (C western region and D eastern region) for 50-year moving intervals in the period 1900–2009. Only climatic factors showing significant response in more than five intervals are shown. Dots represent negative correlation. In uppercase–climatic factors in the year prior to tree-ring formation.

contradictory to the suggested increased sensitivity to drought conditions, in the later part of analysed period there was a positive effect of current year July temperature on TRW (Figs. 4–6) in the eastern region. This could be explained by increased frequency of hot summers coupled with heavy rainfall (Avotniece et al., 2010), which would not necessarily lead to water deficit.

4.3. Earlywood vessel cross-section area

Compared to TRW, EVA had significant correlation and response to a greater number of climatic factors, indicating that EVA can provide additional information on tree response to climate. Temperature in the dormant period had strong effect on EVA, which was less apparent for TRW. The effect of temperature in the cold period most likely is related to tree vigour via damage (presumably to cambium and fine roots) caused by low temperatures (Harvey, 1923; Repo et al., 2008; Steudle, 2000; Tierney et al., 2001). Sensitivity of EVA to temperature in January and February has decreased, as shown by lower correlation in moving intervals after about 1925–1975 (Fig. 5), which can be explained by warmer winters due to climatic change (Briffa et al., 1998; Lizuma et al., 2007). Cold hardening, which in oak proceeds until January (Morin et al., 2007) might be impeded by rising autumn–winter temperatures (Repo et al., 2008), resulting in increased risk of frost damage. The correlation of EVA with January precipitation might be explained by an insulating effect of snow causing reduced depth of frozen soil (Hardy et al., 2001). Thus, a thick snow layer can protect fine roots and decrease their winter mortality (Tierney et al., 2001), indirectly promoting water uptake in spring by a larger surface area of roots. When water uptake is low, smaller vessels are produced to minimise risk of embolism (Cochard and Tyree, 1990; Galle et al., 2010; García-González and Eckstein, 2003; Lovisolo and Schubert, 1998; Tyree and Cochard, 1996).

Correlation with April temperature was significant during the entire analysed period (Figs. 4 and 5), and a significant response of EVA to April temperature is also evident. Considering April as the beginning of wood formation (Ahas et al., 2000; Sass-Klaassen et al., 2011), the significant effect of April temperature on EVA can be explained by direct influence on wood formation (Deslauriers et al., 2008). Low temperature at that time decreases cambial activity (Rossi et al., 2008), which might lead to formation of smaller vessels (Pallardy and Kozlowsky, 2008). The effect of April temperature might potentially weaken in the future due to climate warming (Lizuma et al., 2007), suggested by loss of significant response in recent years (Fig. 6). Positive correlation of temperatures in May–October of the year p.t.f. on EVA may be related with increased amounts of stored assimilates (White et al., 1999), which might promote earlywood formation in the next year (Pallardy and Kozlowsky, 2008; Tardif and Conciatori, 2006).

In the eastern region, an effect of water-deficit in June is suggested by negative correlation of EVA with temperature and positive with precipitation (Fig. 4). However, considering timing of leaf flush of oak in the eastern Baltic region (Ahas et al., 2000; Kalvāne et al., 2009; Sass-Klaassen et al., 2011), vessels have probably formed by the middle of June. Therefore, a water-deficit effect on EVA would only be possible in late-spring years.

5. Conclusion

TRW and EVA showed different climatic signals, which were generally stronger in EVA. Temperatures in winter and spring were found to be the main limiting factors for EVA. TRW was negatively related with July and August temperature in the year prior to tree-ring formation in both regions, likely due to water deficit resulting in decreased assimilation. During the past 100 years, there appears to have been a shift in climate sensitivity of growth oak. An effect of previous year October and winter temperature on TRW has disappeared, but the effect of August precipitation has strengthened. EVA has lost sensitivity to January and February temperature, while the effect of December temperature has strengthened particularly in the more continental eastern region of Latvia.

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