Opinion of the National Committee subgroup on the EURL ECVAM Recommendation on Non-Animal-Derived Antibodies

The EURL ECVAM Recommendation on Non-animal Derived Antibodies

The production of monoclonal and polyclonal antibodies (mAb and pAb, respectively), by using animals has recently been part of a broad scientifically and politically driven debate. The debate was triggered by the Recommendation on Non-Animal Derived Antibodies[1] of the European Commission's Joint Research Centre (EC-JRC) released in 2020, based on the work performed by the Scientific Advisory Committee (ESAC) of the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM).
The EURL ECVAM recommendation urged government authorities, ethical committees, funding agencies and publishers that animals should no longer be used for the development and production of antibodies for research, regulatory, diagnostic, and therapeutic applications. This recommendation was followed by papers from ESAC board members[2,3] claiming that non-animal derived antibodies were ready to replace animal derived ones for all known applications.

Several scientific societies and researchers claimed in response to this that both, the EURL ECVAM recommendation and the published correspondence, contained distorted perceptions of the current possibilities for non-animal-derived antibodies.[4,5,6.]"Insufficient technological development, inconsistent efficiency depending on the application, and difficulty in implementation at a global scale" are named as major concerns barring the substitution of hybridoma technology with animal-free methods[7]. It was also highlighted that some involved ESAC members were having a conflict of interest when advising the EURL ECVAM for the use of non-animal derived antibodies. Moreover, the same *Ad-hoc Members of ESAC* also carried out the *'independent scientific peer review'* as part of the ESAC Working Group, questioning their own scientific standards.

It is undisputable that animal testing should be replaced by alternative methods wherever possible. This is not only a legal requirement in Europe[8] but also in the sense of good scientific practice[9]. This of course also applies for the production of mAb and pAb.

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[1] Viegas Barroso, J., Halder, M. E. & Whelan, M. EURL ECVAM recommendation on non-animal-derived antibodies, EUR 30185 EN (European Union, 2020); https://doi.org/10.2760/80554
[2] Gray, A.C., Bradbury, A.R.M., Knappik, A. *et al.* Animal-derived-antibody generation faces strict reform in accordance with European Union policy on animal use. *Nat Methods* **17**, 755–756 (2020). https://doi.org/10.1038/s41592-020-0906-9
[3] Gray, A. C., Bradbury, A., Dübel, S., Knappik, A., Plückthun, A., & Borrebaeck, C. A. (2020). Reproducibility: bypass animals for antibody production. *Nature*, *581*(7808), 262-263. https://doi.org/10.1038/d41586-020-01474-7
[4] González-Fernández, Á., Bermúdez Silva, F.J., López-Hoyos, M. *et al.* Non-animal-derived monoclonal antibodies are not ready to substitute current hybridoma technology. *Nat Methods* 17, 1069–1070 (2020). https://doi.org/10.1038/s41592-020-00977-5
[5] Gorovits B, Hays A, Jani D, Jones C, King C, Lundequist A, Mora J, Partridge M, Pathania D, Ramaswamy SS, Rutwij D, Shen H, Starling G. AAPS Perspective on the EURL Recommendation on the use of Non-Animal-Derived Antibodies. AAPS J. 2021 Mar 1;23(2):34. doi: 10.1208/s12248-021-00567-z. PMID: 33649990.
[6] European Animal Research Association (EARA)/European Federation of Pharmaceutical Industries and Associations (EFPIA) response t EURL ECVAM recommendation on non-animal-derived antibodies, https://www.efpia.eu/media/580524/eara-efpia-antibody-report.pdf
[7] González-Fernández, Á., Bermúdez Silva, F.J., López-Hoyos, M. *et al.* Non-animal-derived monoclonal antibodies are not ready to substitute current hybridoma technology. *Nat Methods* 17, 1069–1070 (2020). https://doi.org/10.1038/s41592-020-00977-5
[8] Article 4 of Directive 2010/63/EU
[9] e.g. https://www.fraunhofer.de/en/press/research-news/2021/january-2021/avoiding-animal-experimentation.html

European National Committees for the protection of animals used for scientific purposes

In Europe, National Committees[10] for the protection of animals used for scientific purposes have been implemented by each Member State according to Art. 49 of the Directive 2010/63/EU. These National Committees advise competent authorities and animal-welfare bodies, e.g. on the care and use of laboratory animals, and exchange information on project evaluation and best practise in the light of the Directive´s objective of harmonization of legislation. In this sense, the European National Committees meet on a regular basis to exchange information on best practices. At the 3rd informal European NC Network meeting, which took place online in 2021, the EURL ECVAM Recommendation on Non-Animal Derived Antibodies was the focus of an intensive discussion.

It was discussed that a more differentiated picture needs to be presented on the use of non-animal derived antibodies as it was stated in the EURL ECVAM recommendation. Thus, it was decided to form a subgroup of National Committees to follow up on this discussion and to formulate a common opinion on how to deal with the EURL ECVAM Recommendation on Non-Animal-Derived Antibodies. This subgroup was supported by several experts on the field of the production and use of mAb, pAb but also of recombinant Ab (recAb).

The subgroup discussed that guidance is needed on the one hand for researchers to evaluate if the purpose of the research question can be answered by using non-animal derived antibodies or which other method is the most successful one. Competent authorities are also in the need of guidance to evaluate and verify if the use of animals is justified for a specific project purpose. Moreover, it is necessary to identify the scientific and structural issues that are hindering the broad application of non-animal derived antibodies.

The use and production of antibodies

In research, antibodies are important tools for many routine techniques (e.g. flow cytometry, western blots, immunohistochemistry, enzyme-linked immunosorbant assay, immunoprecipitation-mass spectrometry, chromatin immunoprecipitation sequencing)[11], and are thus, an indispensable utensil to answer basic research questions.

Antibodies are also a critical part of medical diagnostics to detect infections, allergies, tumours, hormones or many other biological markers. Moreover, with their ability to bind on almost every target antigen, antibodies are utilised as pharmaceuticals to treat several diseases, e.g. cancer, respiratory diseases, infections, autoimmune diseases, macular degeneration, etc. They can also be used to activate the immune system, are revolutionary in the therapeutic field for different type of tumours (e.g. checkpoint inhibitors), or for passive immunisation (e.g. COVID infection, prevention of tetanus with anti-tetanus immunoglobulins, or preventive/therapeutic antibodies for substitution in immunodeficient patients)[12].

[10] According to Article 49 of the Directive 2010/63/EU https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32010L0063&qid=1687163931612

[11] Koivunen , ME and Krogsrud, RL. Principles of Immunochemical Techniques Used in Clinical Laboratories, *Laboratory Medicine*, Volume 37, Issue 8, August 2006, Pages 490–497, https://doi.org/10.1309/MV9RM1FDLWAUWQ3F

[12] Varadé, J., Magadán, S. & González-Fernández, Á. Human immunology and immunotherapy: main achievements and challenges. *Cell Mol Immunol* **18**, 805–828 (2021). https://doi.org/10.1038/s41423-020-00530-6

Many different commercial and in-house developed antibodies are being used for different purposes, mostly research, diagnosis, purification of compounds and therapy. As recently shown by the COVID-19 crisis, the medical benefit of diagnostic and therapeutic antibodies for patients has reached such a level in recent years that modern medicine can no longer be imagined without them. Antibodies can be received in different ways and each of the techniques have their advantages and limitations.

Polyclonal antibodies (pAB). The production of pAb involves the use of different species (e.g. rabbits, goats, horses, chickens, camelids etc.) that have been exposed to an antigen evoking the respective immune response. pAb can also be derived by volunteering humans, which were either immunized by vaccination or were recovering from infections. The blood sera containing the pAb have multiepitope binding properties and can be used either directly or further be purified by affinity techniques. In the case of chickens, polyclonal immunoglobulin Y (IgY) can be purified from the egg even without invasive blood sampling[13]. pAb are marketed for all kinds of diagnostics from autoimmune to infectious diseases or from point of care to clinical testing. In addition, they are indispensable for the treatment of certain clinical conditions like tetanus or snakebites. In the therapeutic field, they can also be used for several purposes[14] including prevention of infections for those patients with humoral immunodeficiencies, treatment for Ebola[15] or COVID-19 infections, autoimmune disorders, and Kawasaki syndrome in children[16]. pAb can be produced within a short period of time and allow for antigen recognition even if some of the epitopes are not accessible. Disadvantages of pAb are the high batch-to-batch variability and the increased likelihood to cross-react with other proteins[17]. Thus, they are of election as secondary reagents on immunological techniques.

Monoclonal antibodies (mAb). mAb are directed to a specific antigenic determinant and can be obtained by means of the hybridoma technology[18]. This technique usually involves the merging of mortal, antibody-producing B-cells from the spleen (or from other sources such as lymph nodes) from immunised animals with immortal myeloma cells. For the production of mAb using the hybridoma technique, a limited number of animals is needed. If the immunization and fusion is successful, only one animal is required. In general, from three to five animals are immunized and sacrificed to generate a hybridoma specific for a single antigen. The hybridomas secreting antigen-specific antibodies are then selected by screening against the antigen of interest, which is relevant for the desired application. Even human mAb can be gained by using hybridomas from transgenic humanized mice or other types of humanized animals (e.g., immunodeficient mice carrying human bone marrow cells). This can be particularly useful, if B-cells from immunized human donors are not available for cell fusion. Selected and stable hybridomas can be maintained on culture or frozen for long periods of time. These hybridomas form a long-term source of antibody producing cells without the need of further animals. General advantages of mAb over pAb are, e.g. the high homogeneity, the possibility to produce a large

[13] León-Núñez et al., Antibodies 2022, 11, 62. https://doi.org/10.3390/antib11040062

[14] Kóhler, G and Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495–497 (1975). https://doi.org/10.1038/256495a0

[15] Ciencewicki JM et al, Characterization of an Anti-Ebola Virus Hyperimmune Globulin Derived From Convalescent Plasma, *The Journal of Infectious Diseases*, Volume 225, Issue 4, 15 February 2022, Pages 733–740, https://doi.org/10.1093/infdis/jiab432.

[16] Perez, E. E. et al. Update on the use of immunoglobulin in human disease: a review of evidence. *J. Allergy Clin. Immunol.* **139**, S1–S46 (2017). doi: 10.1016/j.jaci.2016.09.023.

[17] https://www.meticulousresearch.com/product/veterinary-diagnostics-market-5123

[18] Kóhler, G and Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495–497 (1975). https://doi.org/10.1038/256495a0

amount of identical antibodies, the high affinity to a single epitope with a low cross-reactivity.[19,20] In addition, mAb have a lower batch-to-batch variation than pAb[21] and, thus, are more likely to entail a better reproducibility of research performed with the respective antibody. Moreover, they are opted to humanization, which allows for better therapeutic applications.

Recombinant antibodies (recAb). Nowadays, antibodies can also be produced in different ways by means of genetic engineering. One major advantage of recAb is that they can be modified with various methods. For example, antibodies derived from animals can be humanized, individual antibody fragments (like Fab/Fc, single chain Fv, etc.) can be generated, or the formation of antibodies with a different isotype or glycosylation pattern as well as the addition of drugs is possible[22].

- *recAb by re-cloning from hybridoma.* The sequences of the heavy and light antibody chains expressed by the hybridoma lines can be determined. This information can be used for the recombinant production of mAb in well-established mammalian expression systems[23]. Under the aspect of quality assurance, this approach has technical advantages over the use of hybridoma-produced mAb. In a certain percentage of cases, additional light and/or heavy chains might be present in the hybridoma cell lines, which might give rise to additional antibodies produced by a hybridoma line, resulting in oligoclonal mixtures of antibodies instead of single-specific mAbs.[24]
- *recAb by display techniques (e.g., phage display)*[25]. A prerequisite for this technique is to generate or buy libraries consisting of a large variety of antibody fragments. Phage antibody libraries can be classified as naïve, i.e. produced from natural antibody donors (humans or non-immunized animals), immune, i.e. cloning antibodies from immunised (transgenic) animals or diseased or vaccinated humans, or as semi-synthetic or synthetic (animal-free) libraries. For the cloning of the antibody fragments, mRNA must be isolated from hybridoma, spleen or lymphatic cells, which is then converted to cDNA. These antibody fragments are combined with bacteriophage coat proteins, so the bacteriophages will display the antibody fragments on their surface. Subsequently, the phages are panned on a solid phase to identify those phages with the highest affinity for the antigen. The antibody genes from the selected bacteriophage are cloned into appropriate vectors to generate a full antibody or other types of antibody fragments. Human mAb can also be obtained using the phage display techniques combined with genetic engineering.[26] It is also possible to produce multiclonal antibodies binding to different epitopes, which might be useful for certain applications.

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[19] Parray HA, et al. Hybridoma technology a versatile method for isolation of monoclonal antibodies, its applicability across species, limitations, advancement and future perspectives. Int Immunopharmacol. 2020 Aug;85:106639. doi: 10.1016/j.intimp.2020.106639.

[20] Mitra S, Tomar PC. Hybridoma technology; advancements, clinical significance, and future aspects. J Genet Eng Biotechnol. 2021 Oct 18;19(1):159. doi: 10.1186/s43141-021-00264-6.

[21] Lipman NS, Jackson LR, Trudel LJ, Weis-Garcia F. Monoclonal versus polyclonal antibodies: distinguishing characteristics, applications, and information resources. ILAR J. 2005;46(3):258-68. doi: 10.1093/ilar.46.3.258

[22] Jin S, Sun Y, Liang X, Gu X, Ning J, Xu Y, Chen S, Pan L. Emerging new therapeutic antibody derivatives for cancer treatment. Signal Transduct Target Ther. 2022 Feb 7;7(1):39. doi: 10.1038/s41392-021-00868-x

[23] Fliedl L, Grillari J, Grillari-Voglauer R. Human cell lines for the production of recombinant proteins: on the horizon. N Biotechnol. 2015 Dec 25;32(6):673-9. doi: 10.1016/j.nbt.2014.11.005.

[24] Bradbury A et al., MAbs. 2018 May/Jun;10(4):539-546. doi: 10.1080/19420862.2018.1445456.

[25] Sharon, J., Sompuram, S.R., Yang, CY., Williams, B.R., Sarantopoulos, S. (2002). Construction of Polyclonal Antibody Libraries Using Phage Display. In: O'Brien, P.M., Aitken, R. (eds) Antibody Phage Display. Methods in Molecular Biology™, vol 178. Humana Press. https://doi.org/10.1385/1-59259-240-6:101

[26] Khan F H Chapter 25 - Antibodies and their applications In: Animal Biotechnology (Second Edition) Academic Press, Editors: A S Verma, A Singh 2020, 503-522, ISBN 9780128117101, https://doi.org/10.1016/B978-0-12-811710-1.00023-9

- *recAb by single cell sequencing.*[27] B-cell screening techniques, allow for the isolation of blood antigen-specific antibodies from blood B-cells obtained from immunised humans (either infected or vaccinated).[28] However, this methodology is not always applicable due to self-tolerance to human components or ethical concerns.

Despite the different techniques, it is important that the resulting antibody must be of the highest quality and needs to be thoroughly validated for the targeted application. Failure to validate the antibody for the intended application leads to a lack of reproducibility of experimental results and suboptimal data quality, undermining the original research goal.[29]

Opinion of the National Committee subgroup

The signing National Committees and authors of this document concluded that the EURL ECVAM recommendation does not provide a balanced information on the limitations of non-animal derived antibodies. So far, scientific evidence is missing that non-animal derived antibodies can fully replace the hybridoma technology and polyclonal animal sera. There are theoretical and practical limitations to all current techniques for the development of mAb and pAb. Thus, an uncritical and full application of the recommendation during the approval process of animal experiments in Europe could create a serious hindrance to the future development of antibodies as diagnostics, in research, for purification of compounds and as therapeutics.

Members of the ESAC board also recently acknowledged that "a restriction on the use of immunization today, without substantial efforts to improve general access to non-animal-derived antibodies, would indeed significantly hamper research". In addition, they proposed that, especially for drug development, the best and most successful approach would be the simultaneous use of different methods (including immunisation) to be most successful. At the same time, the authors promote to improve the access to non-animal derived antibodies.[30] The European Commission also stressed that the EURL ECVAM Recommendation on Non-Animal-Derived Antibodies does not propose a ban on the use of animals for the development and production of antibodies *per se.* The Directive 2010/63/EU requires a case-by-case scrutiny of project proposals to ensure that only when there is robust and legitimate justification for the use of animals a project should be authorised.[31]

However, it remains unclear, how this justification should be provided by researchers in their specific field in cases in which information on the direct comparison of efforts and results using the different approaches – recombinant *versus* classical hybridoma strategies – is missing. Therefore, care should be taken not to generalize statements obtained on individual antigens or antibody types to the diverse range of potential antigens and applications.

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[27] Pedrioli A, Oxenius A. Single B cell technologies for monoclonal antibody discovery. Trends Immunol. 2021 Dec;42(12):1143-1158. doi: 10.1016/j.it.2021.10.008.

[28] Pedrioli A and Oxenius A, Single B cell technologies for monoclonal antibody discovery, Vol. 42, 12. Trends in Immunology, 2021, DOI:https://doi.org/10.1016/j.it.2021.10.008

[29] Weller MG. Ten Basic Rules of Antibody Validation. *Analytical Chemistry Insights*. 2018;13. doi:10.1177/1177390118757462

[30] Andrew R.M. Bradbury, Stefan Dübel, Achim Knappik & Andreas Plückthun (2021) Animal- versus in vitro-derived antibodies: avoiding the extremes, MAbs, 13:1, doi: 10.1080/19420862.2021.1950265

[31] See the FAQs on the EURL ECVAM Recommendation on non-animal derived antibodies, released in 2022, https://joint-research-centre.ec.europa.eu/eu-reference-laboratory-alternatives-animal-testing-eurl-ecvam/eurl-ecvam-faqs/frequently-asked-questions-eurl-ecvam-recommendation-non-animal-derived-antibodies_en

Within the subgroup there was a common understanding that, at the moment, all technologies are complementary[32], and researchers and companies should choose the most appropriate method depending on their purposes, analysing their advantages and disadvantages.[33] The authors and signing National Committees of this document strongly believe that all current technologies of antibody discovery platforms including existing hybridoma cell lines, phage display and single B cell technologies[34] have their merits and should be used depending on the research question, target of interest, and research experience.
However, the authors and signing National Committees of this document also speak out in favour that non-animal derived antibodies should be considered and be used if they are suitable and where they are demonstrated to be at least equivalent or better to address the specific research question.
A commitment exclusively to the use of non-animal derived antibodies is premature. Within the EU this could mean competitive disadvantages in basic research, diagnostics and therapeutics considering the continuation of traditional hybridoma-based mAb production outside the legislation sphere of the EU, and already drives researchers to countries with less stringent standards on animal protection.

Measures to increase the uptake of non-animal derived antibodies

The authors and signing National Committees agree with the EURL ECVAM recommendation that several measures should be established to increase the uptake of non-animal derived antibodies. As a first step, the infrastructure should to be changed to support a broader implementation of non-animal antibody generation techniques. The access to training courses for researchers at different levels of their career should be alleviated so researchers become aware about the possibilities of non-animal derived antibodies and can learn about the genetic engineering techniques at a low cost. In addition, better and affordable access to non-animal derived antibodies and phage display libraries could pave the way to a broader implementation of recAb.

Measures should also include the installation of dedicated national and EU-wide research programmes and/or implementing academic core facilities to compare the different approaches on a broad range of different antigens and applications. A dedicated funding instrument on European level would be most helpful to bring together the experts on classical mAb/pAb technologies and recAb approaches and to accumulate the best knowledge represented in both worlds. Research directly comparing mAb technology with phage display libraries against the same antigen should be encouraged and the results of such investigations should be publicly available.
Following points should be considered by policy makers, academia, non-academic institutions, funders and companies:

1. *In vivo* and *in vitro* techniques to produce Ab are complementary approaches, each with pros and cons and should be used depending on the research question, target of interest, and research experience. For specific purposes of interest, institutions and funders could support the use of *in vivo* and *in vitro* techniques in parallel. Thereby, knowledge on advantages and

[32] Rossant, C. J. et al. *MAbs* 6:6, 1425-1438, DOI: 10.4161/mabs.34376
[33] in accordance to Article 38 paragraph 1 (b) of Directive 2010/63/EU: the purposes of the project justify the use of animals
[34] Jane Zveiter Moraes et al, Hybridoma technology: is it still useful?, Current Research in Immunology,2: 32-40 (2021) https://doi.org/10.1016/j.crimmu.2021.03.002.

limitations of each technique will be increased and fields could be identified where the non-animal methods are leading to better results.

2. The broad uptake of non-animal derived Ab is partially hindered by a low availability of phage display libraries and practical scientific knowledge on how to select optimal recombinant mAbs for a dedicated research goal. Ways should be found to make high quality phage display libraries freely accessible, especially for small research groups, and to introduce training curricula on a non-profit basis. Joint core facilities could be established providing researchers from different institutions with the desired non-animal derived antibody.

3. Researchers should be supported in using *in vitro* approaches. Thus, the technology developing Ab via phage display should be part of the curriculum or implemented in other teaching programs. This should include the use of both non-immune and immune libraries to obtain scientifically sound and high-quality results. The time and resources (personnel, consumables) necessary to bring this forward need to be allocated by the institutions and funding agencies to support these comprehensive efforts.

MEMBERS OF THE NATIONAL COMMITTEE SUBGROUP

- **GERMANY**
  - **Bettina Bert**
    - German National Committee representative, German Federal Institute for Risk Assessment (BfR), German Centre for the Protection of Laboratory Animals (Bf3R), Max-Dohrn-Str. 8-10, 10589 Berlin, Germany
    - email: bettina.bert@bfr.bund.de
  - **Prof. Dr. Thomas H. Winkler**
    - Nikolaus-Fiebinger Centre for Molecular Medicine of the University of Erlangen-Nuremberg, Germany
    - email: thomas.winkler@fau.de
  - **Prof. Dr. Manfred B. Lutz**
    - University of Würzburg, Institute for Virology and Immunobiology. Versbacher Str. 7, 97078 Würzburg, Germany)
    - email: manfred.lutz@uni-wuerzburg.de
- **HUNGARY**
  - **Dr. István Gyertyán.**
    - Pharmacology and pharmacotherapy, Semmelweis University, Budapest, Hungary
    - email: gyertyan.istvan@med.semmelweis-univ.hu; atet@nebih.gov.hu
  - **Prof. Dr. Péter Balogh**
    - Immunology and Biotechnology department. University of Pecs. Szigeti ut 12, H7634, Hungary
    - email: balogh.peter@pte.hu
- **NETHERLANDS**
  - **Dr. Erwin L. Roggen**
    - CEO 3Rs Management and Consulting ApS, Lyngby, Denmark, representing the Danish 3R Center and National Committee
    - email: ELRo@3rsmc.onmicrosoft.com
  - **Bea Zoer**
    - GD Animal Health. Eindhoven, Noord-Brabant, Netherland
    - email: bea.zoer@rvo.nl
  - **Dr. Wim A. Deleeuw**
    - Head Animal Welfare Body Utrecht University
    - email**:** w.a.deleeuw@uu.nl
- **FRANCE**
  - **Dr. Thomas Bouquin:**
    - Head of Global Antibody Research Platform, Large Molecule Research, SANOFI
    - email: Thomas.Bouquin@sanofi.com
- **LUXEMBOURG**
  - **Vicky Binck:**
    - Veterinarian at Ministry of Agriculture, Luxembourg.
    - email: Vicky.BINCK@alva.etat.lu

- **BELGIUM**
  - **Prof. Dr. Alban de Kerchove D´Exaerde**
    - Lab Neurophysiology, ULB Neuroscience Institute, Université Libre de Brussels (ULB), Belgium
    - email: alban.de.kerchove@ulb.be
  - **Reineke Hameleers**
    - CEO Eurogroup for animals. Brussels, Belgium
    - email: r.hameleers@eurogroupforanimals.org
- **SWEDEN**
  - **Kaisa Askevik.**
    - Administrative officer at the Swedish 3R Centre på Jordbruksverket.
    - email: Kaisa.Askevik@jordbruksverket.se
  - **Prof. Leif Carlsson.**
    - UMEA University, Sweden
    - email: leif.carlsson@umu.se; 3Rcenter@jordbruksverket.se
- **SPAIN**
  - **Prof. Dr. África González Fernández**
    - Spanish NC representative from Ministerio de Agricultura, Pesca y Alimentación. Spain. University of Vigo, CINBIO, Campus Universitario de Vigo. 36310 Vigo,
    - email: africa@uvigo.es