# Mosquito Irradiation, Sterilization and Quality Control

*Report of the First Research Coordination Meeting*
*of an FAO/IAEA Coordinated Research Project,*
*held in Vienna, Austria,*
*from May 31 to June 4, 2021 (Virtual)*

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## Summary:

The application of the Sterile Insect Technique (SIT) in area-wide integrated pest management (AW-IPM) programmes continues to increase in response to requests from Member States. These requests include the development and refinement of SIT packages for programmes to control populations of different insect pests of agricultural, veterinary and human health importance. The development and operational application of such programmes with an SIT component against human disease vectors depends on the efficiency of irradiated sterile males to induce sterility in the target populations. Irradiation, in combination with other steps in the workflow from production to release of the sterile males is a critical point ultimately affecting performance in the field. Quality control is critical at every step of the SIT workflow, both pre- and post-irradiation.

This CRP will be the opportunity to investigate the impact of endogenous factors such as stage, age and genetic background on irradiation efficacy, as well as physiological mechanisms associated with genetic variation in radiation responses. We will also explore the impact of exogenous factors on radio-sensitivity, such as temperature, density, oxygen availability, irradiation source, dose-rate and energy of the rays. Another important aspect to characterize is the impact of irradiation on vectorial capacity of females, as well as on the cytoplasmic incompatibility and pathogen interference conferred by *Wolbachia* infection.

Finally, it will be important to develop and validate new quality control tools to monitor the product quality along the production chain and compare it between production and release centres.

## Background

Scientific situation and problems to be researched: Insects are the most abundant, speciose and diverse animal group on this planet. Although most insect species are beneficial or harmless, there are a select few which are vectors of human diseases and their populations need to be managed. Conventional control methods are primarily based on insecticides. However, there are increasing concerns about their negative impact on human health and environment, as well as the inevitable selection of insecticide resistance due to their extensive use. The Sterile Insect Technique (SIT) represents a species-specific, non-polluting and environment-friendly approach that has been extensively used over the last 60 years to control populations of plant and animal pests, and animal disease vectors as a component of AW-IPM programmes. Due to its successful use against different target species, the requests for the application of the SIT against human disease vectors continues to increase from FAO and IAEA Member States (MS). Programme efficiency and cost-effectiveness depend on the efficiency of irradiated sterile males to induce sterility in the target populations. Irradiation, in combination with other steps in the workflow from production to release of the sterile males, is a critical point ultimately affecting performance in the field. Quality control is also critical at every step of the SIT workflow.

*Targeted species*: Currently,the following species of human health importance are considered potential targets for the SIT: *Aedes aegypti, Ae. albopictus, Ae. polynesiensis, Anopheles albimanus, An. arabiensis, An. darlingi, An. gambiae* complex*, An. stephensi, Culex pipiens* complex.

Importance of irradiation and quality control in SIT programmes

Historically, a variety of chemosterilants were used to sexually sterilize male mosquitoes with varying success and suitability for larger scale SIT programmes, and the evaluation of sterilizing male mosquitoes by irradiation has suggested that this is, to date, the most practical, safe and environment-friendly way to induce sterility, especially at large scale [Helinski et al., 2006]. The use of isotopic sources for gamma radiation (usually cobalt-60 or caesium-137), has been most commonly used for AW-IPM programmes with an SIT component. However, X rays and high energy electrons (in this case "high" refers to a minimum of 1-5MeV) are now becoming viable and practical alternatives (Gómez-Simuta et al., 2021). In irradiation processes, the key factor is the absorbed dose, which needs to be accurately controlled to ensure that treated insects are rendered sufficiently sterile but are still able to compete for wild females upon release. Therefore, accurate dosimetry (measurement of absorbed dose) is critical. Factors such as insect age and stage, handling methods, oxygen level, ambient temperature, dose-rate and many others prior to- and during irradiation, influence both the radio-sensitivity and biological viability of the irradiated mosquito. Because of these sources of variation, there is presently a lack of consensus on the irradiation protocols to produce high quality sterile male mosquitoes for field release, especially at a large scale. Thus, exploring the impact of these different factors, and their impact on the quality of the resulting sterile males, in interaction with other handling steps before release, is essential. A careful evaluation of these factors in the design of irradiation protocols can help to find a balance between the sterility and competitiveness of the irradiated males destined for field releases.

Many SIT programmes apply higher doses than required as a "precautionary" measure to ensure full sterility. However, this is likely to decrease the overall competitiveness of the sterile males which could compromise their effectiveness in the field. Therefore, the studies in this CRP proposal aim to understand the various factors affecting dose-response in mosquitoes to standardize the irradiation processes to optimize male sterility and quality.

Moreover, the quality of the mosquitoes should be monitored with standard quality control (QC) procedures throughout the production process of the sterile males. The QC is necessary to both assess the efficacy and efficiency of mass-rearing and to predict the performance of those insects. There is a need to validate existing QC tests for both mass-rearing and field performance. Therefore, the studies in this CRP proposal aim to develop two types of QC indicators:

- Quick and efficient parameters that will be monitored routinely;
- More labour-intensive parameters that will be measured periodically over longer time intervals.

## CO-ORDINATED RESEARCH PROJECT (CRP)

This Coordinated Research Project (CRP) is based on an expert group meeting on "Harmonization of Irradiation and Dosimetry Protocols for *Aedes* Invasive Mosquitoes" conducted in February 2018 and May 2019, Vienna, Austria.

The overall objective of this new **CRP D44004-CR-1**, approved for the **period 2020-2025**, is to understand irradiation induced effects, endogenous and exogenous factors that affect or improve dose-response, irradiation dosimetry procedures, impact of irradiation on vectorial capacity, impact of irradiation on cytoplasmic incompatibility and pathogen interference by *Wolbachia*, and to develop tools to assess quality of sterile male mosquitoes.

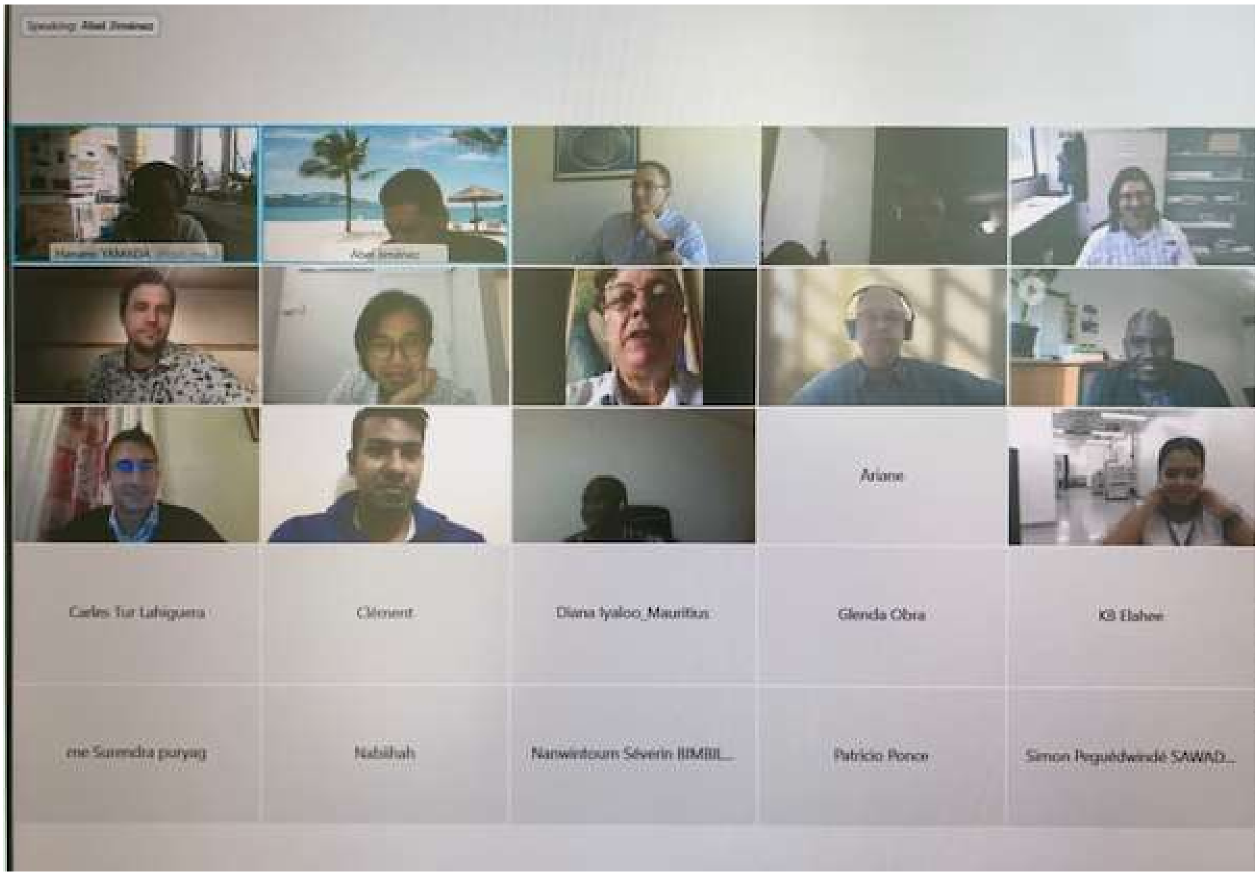
## FIRST RESEARCH CO-ORDINATION MEETING (RCM)

21 scientists from 19 countries (including hosts) presented virtually during this first RCM, organized from Vienna, Austria from May 31st to June 4th, 2021. The list of participants, which included CRP contract and agreement holders is given in **Annex 1**. The agenda for the meeting is attached in **Annex 2**.

During the first three days of the RCM meeting, participants presented research relevant to the CRP, as well as their research plans for the following 18 months of the CRP.

During the last two days of the meeting, general discussions were held to define and review the thematic areas of the CRP, the review of the general and specific R&D objectives to be addressed during the 5 years of the CRP, and the CRP Logical Framework, in order to agree on minimum outputs to be achieved at the end of the CRP.

Abstracts of the presentations are compiled in **Annex 3** and a copy of all presentations were made available to all participants at the end of the RCM.



## Individual Plans according to topics

### Irradiation & Sterilization

| Sub-topics: | Researchers | main interests |
|---|---|---|
| Irradiator qualification | Florent Kuntz | dosimetry; irradiators |
| | Patricio Ponce | *mosquito organ depth, sperm production/age* |
| | Hadian Sasmita | *dosimetry; SOP e-beam* |
| | Carlos Tur Lahiguera | *dosimetry; e-beam* |
| Factors affecting dose-response | Dan Hahn | *hypoxia; stress factors* |
| | Carlos Tur Lahiguera | *various factors* |
| | Hadian Sasmita | *dose-rate; starvation* |
| | Glenda Obra | *various factors* |
| | Romeo Bellini | *pupae densities* |
| | Ambicadutt Bheecarry | *strain origin;* $Cs^{137}$ *vs* $Co^{60}$ |
| | Louis Clement Gouagna | *endo. exo. factors* |
| | Jair Virginio | *pupae densities (atm); adult/pupae* |
| Effects of irradiation on vector competence | | |
| | Ariane Dor | *transm. risk released females* |
| | Simon Sawadogo | *irrad. An. arab.* |
| | Clement Gouagna | Chik. Deng, Albo |
| | Pattamaporn Kittayapong | *Ae. aegypti* |
| Effect of irradiation on symbionts | Pattamaporn Kittayapong | *Ae. aegypti* |

## Quality Control

| Sub-topics: | Researchers | main interests |
|---|---|---|
| Product Quality control | Carlos Tur Lahiguera | *product QC* |
| | Ariane Dor | *estab. baseline QC param.; novel tools* |
| | Thierno Bakhoum | *QC test validation* |
| | Ambicadutt Bheecarry | *QC test validation* |
| | Antonios Michailakis | *novel QC tests* |
| | Glenda Obra | *QC test validation* |
| | Simon Sawadogo | *An. arab. Swarming dynamics; optimize use of rhod.B for mating tests* |
| | Zhiyong Xi | *product QC* |
| | Louis Clement Gouagna | use of rhod.B field compet |
| Process quality control | | |
| | Carlos Tur Lahiguera | *automated systems; process e-beam* |
| | Ariane Dor | *novel tools* |
| | Hadian Sasmita | *process e-beam irrad* |
| | Michael Samuel | *irrad en masse Anoph.* |
| | Kajla Seheli | *irrad. en masse; UPSCALING PROCESS* |
| | Louis Clement Gouagna | *irrad protocols* |
| Factors affecting downstream sterile male quality | | |
| | Arinae Dor | *effects of chilling* |
| | Michael Samuel | *handling/irrad. methods* |
| | Dan Hahn | *cold tolerance* |
| | Heath MacMillan | *cold tolerance* |

| | |
|---|---|
| Simon Sawadogo | *temp, dose on dispersal & comp; swarming* |
| Glenda Obra | *irrad effects on dispersal* |
| Zhiyong Xi | *factors improve irrad. outcome for females* |

## 1. Irradiation & Sterilization

Irradiation and dosimetry procedures

Standard dosimetry procedures using Gafchromic® film dosimeters for SIT are presented in **(IAEA, 2004)** and ISO standards are available for the use of radiochromic films (ISO/ASTM 2011) and dosimetry for SIT programmes (ISO/ASTM 2013). However, there is a need to develop standardized protocols for both irradiation and dosimetry procedures for human disease vectors.

Endogenous factors on irradiation efficacy

Life stage and age within each life stage are known to impact radiation sensitivity in many insect taxa, with radiation sensitivity decreasing with increasing developmental stages i.e. adults are less susceptible to radiation than larvae **(Cogburn, Tilton and Brower, 1973; Dey and Manna, 1983; Dongre *et al.*, 1997; Hallman and Thomas, 2010; Williamson, Mitchell and Seo, 1985)**. Similarly, it is well known that some insect taxa are more susceptible to sterilization by radiation than others **(Bakri *et al.*, 2005; Bakri, Mehta and Lance, 2005)**. For example, it takes much higher doses of radiation to sterilize moths than mosquitoes. It is not surprising that highly divergent insect taxa vary in their radiation sensitivities, but there is some limited evidence for radiation susceptibility differing between populations **(Azizyan and Ter-Hovhannesyan, 2010)**. Terzian and Stahler **(Terzian and Stahler, 1966)** showed that radiation tolerance increases in eggs of the mosquito *Aedes aegypti*. Specifically, they showed substantially greater survival of mosquitoes after egg irradiation in a line selected over 35 generations compared to the unselected parental colony that had been in the laboratory for more than 20 years. Thus, we know there can be heritable variation for radiation susceptibility segregating even within long-standing laboratory colonies. Similarly, there is a substantial body of literature that shows naturally segregating genetic variation in radiation susceptibility within populations of the fly Drosophila melanogaster **(Enfield, North and Erickson, 1981; Westerman and Parsons, 1973)**.

In addition to genetic variation, there may be other types of heritable effects on radiation sensitivity in insects due to epigenetic changes or vertically transmitted microbes. Beyond intracellular bacteria that are clearly vertically inherited (see specific paragraph on *Wolbachia* below), other microbiota may impact radiosensitivity by modulating the immune system which warrants more research.

Exogenous factors on irradiation efficacy

Mass-rearing and diet (nutritional state) affects mosquito overall biological quality, and may also affect dose-response, or more likely the propensity to repair cellular/DNA damage during and following irradiation procedures. Handling procedures prior to- and during irradiation may also have an effect on the resulting sterile adult male biology. Handling procedures include variable factors such as irradiation medium (in water or air), or pupal densities inside the sample irradiated **(Yamada *et al.*, 2019)**.

It has been suggested that lowering the ambient temperature during irradiation treatments reduces radiosensitivity, by reducing the insects' metabolic rate **(Rananavare, Harwalkar and Rahalkar, 1991)**. This has yet to be assessed and confirmed for mosquito pupae, however maintaining consistency in all irradiation experiments in terms of temperature is good practice- not only for sterilizing pupae, but also for consistency and reliability in the dosimetry applied.

The oxygen levels, i.e. the atmospheric condition in which mosquitoes are subjected to before, and during radiation exposure can greatly influence the resulting induced sterility following irradiation, as is seen in other insects. Radiation effects are generally reduced in oxygen-poor environments (hypoxia) as compared to relatively oxygen-rich environments, or normoxia. Normoxia is defined as having roughly the same atmospheric oxygen content as would be expected in the local atmosphere at normal pressure and temperature, where normal temperature is 20°C and pressure is 1 atmosphere. Gaseous oxygen levels are often estimated as the partial pressure of oxygen relative to the other gases in the atmosphere, but for insects in regular air the field often approximates this as a percentage of oxygen relative to other gases in that air sample if at near normal pressure and temperature conditions. For example, the oxygen content of atmospheric air at normal pressure is often approximated as 21%. While a hypoxic condition is technically any atmosphere that is less than 21% oxygen, insects do not typically react to hypoxic conditions until oxygen levels reach less the 16% and hypoxia does not generally acutely affect insect growth, performance, and reproduction until oxygen content falls below 6% - termed severe hypoxia **(Harrison, Greenlee and Verberk, 2018)**. There is a substantial literature showing that exposure of insects to severe hypoxia prior to and during irradiation affects the insects response to radiation, including lower levels of induced sterility or mortality for a given dose in severe hypoxia as well as occasionally improved post-irradiation performance of sterile males. Thus, the availability of oxygen to mosquito pupae and adults prior to and during the irradiation process should be carefully considered as a parameter that could affect the outcomes of the radiation treatment.
The effects of irradiation source, dose-rate and energy on dose-response in insects are not well understood. Their effects are often difficult to assess as researchers seldom have multiple irradiation devices to directly compare their sterilizing efficiency while controlling all (or most) other internal and external factors. Therefore, little data exists regarding this variable

<u>Impact of male residual fertility on target populations</u>
The impact of the potential introduction of colony genotypes and irradiation induced mutations into targeted populations through male residual fertility (e.g. possible effect on vectorial capacity) has not been studied. While it is known that optimal irradiation doses, designed to achieve the best combination of competitiveness and sterility, usually confer sub-sterility, no information is available on the traits of the produced progeny, which is relevant in the case of mosquitoes.

<u>Impact of irradiation on vector capacity</u>
When considering a mosquito release programme, one of the primary issues to be addressed is to the elimination of the females since females are blood feeders and therefore are potential disease vectors. However, current sex sorting techniques are not 100% efficient, and a small number of females are dispersed with males during releases. For this reason, the impact of irradiation on vectorial capacity needs to be assessed to determine at which level irradiated female mosquitoes can still transmit disease pathogens.
It has been shown that irradiated females of several species of mosquitoes did not produce eggs at doses lower than those required for male sterilization (for *Anopheles arabiensis*, **(Poda *et al.*, 2017)**; **(Dandalo *et al.*, 2017)**, *Aedes albopictus*, **(Balestrino *et al.*, 2010; Bond *et al.*, 2019; Yamada *et al.*, 2014**; Damiens personal communication), and *Ae. aegypti*,

**(Bond *et al.*, 2019)** but no effect on blood feeding behaviour or survival in the laboratory has been observed for *Anopheles arabiensis* **(Dandalo, 2017)** and for *Aedes albopictus* **(Moretti *et al.*, 2021;** Damiens personal communication). However, for *Aedes aegypti*, a reduction of blood feeding and longevity has been observed **(Aldridge *et al.*, 2020)**. Further experiments are needed to study this potential impact of irradiation. A modification of blood feeding or survival will have a strong effect on vectorial capacity.
Moreover, direct effects of irradiation on vector competence should also be assessed to determine if irradiated female mosquitoes can transmit disease pathogens. In a trial conducted in Burkina Faso on *Anopheles arabiensis*, oocyst prevalence of *Plasmodium falciparum* was significantly reduced in irradiated females but oocyst intensity (mean number of oocyst in the midgut of infected females) was not affected by irradiation **(Guissou *et al.*, 2020)**.

<u>Impact of irradiation and mass-rearing on cytoplasmic incompatibility and pathogen interference conferred by *Wolbachia*</u>

The combined IIT/SIT approach has been proposed to minimize the possibility of unintended population replacement, in which low-dose irradiation is used to sterilize any residual females not removed from released male mosquitoes without affecting the latter's fitness or mating performance **(Zheng *et al.*, 2019)**. In these combined IIT/SIT strategies, cytoplasmic incompatibility between Wolbachia-infected males from the colony with wild females not infected with the same Wolbachia strain plays the primary role in population suppression in the release program **(Zhang *et al.*, 2015)**. While some have suggested that IIT induced by *Wolbachia* or other vertically transferred microbes is sufficient as a mechanism for suppressing mosquito populations, the potential for accidental release of *Wolbachia*-infected females that could in turn cause the novel *Wolbachia* to be established in the environment is substantial when considering the release of millions of sterile male insects **(Zhang *et al.*, 2015)**. Thus, radiation treatments are often combined with *Wolbachia*-based IIT to sterilize the few females that are released during a large-scale operational program. In this case of IIT/SIT combined technique, the goal is to select doses that will completely sterilize females, but not necessarily sterilize males – although the additional partial sterilizing effects of radiation on males may also be useful for population suppression.
Intracellular endosymbiotic bacteria in the genus *Wolbachia* are well known for their many physiological effects on insects, from inducing reproductive incompatibilities to altering vector competence for a number of viruses that can cause human diseases, among others **(Dutra *et al.*, 2016; van den Hurk *et al.*, 2012; Pan *et al.*, 2017**, etc.). To our knowledge, there are no published studies evaluating the extent to which infection with *Wolbachia* or the particular strain of *Wolbachia* used for infection may affect the dose of radiation needed to sterilize females. It is possible that some *Wolbachia* strains may have greater effects on radiation sensitivity than other strains due to the physiological mechanisms that they may induce. Furthermore, it is also possible that the effects of any particular *Wolbachia* strain on mosquito physiology may be dependent on the genetic background of the mosquito strain involved. *Wolbachia* infection has been clearly shown to affect mosquito physiology with respect to the immune system and vector competence for a wide range of viruses, thus there may be additional interactions between radiation dose responses, *Wolbachia* genetic background, and the genetic background of the mosquito colony that require further investigation.

The *Aedes albopictus* line ”HC” is superinfected with three different strains of *Wolbachia*: the original native double-infection involving wAlbA and wAlbB, and the transinfected wPip **(Zheng *et al.*, 2019)**. The wPip strain originated from the mosquito *Culex pipiens* and induces complete unidirectional cytoplasmic incompatibility (CI) when the triple-*Wolbachia*-infected HC males mate with wild-type *Ae. albopictus* females carrying only the native wAlbA and wAlbB double-infection. Given that IIT is based on CI conferred by *Wolbachia*, it is necessary to assess the impact of irradiation or mass-rearing on cytoplasmic incompatibility. Longitudinal monitoring every three months indicated no impact of mass-rearing on cytoplasmic incompatibility in *Aedes albopictus* HC line on CI (Y. Wu, pers. com.). wPip in HC mosquitoes inhibited replication of both Zika and dengue virus and blocked both horizontal and vertical transmission of Zika virus, indicating its ability to generate *Wolbachia*-mediated viral blocking. Data of annual monitoring indicate no impact of mass-rearing on pathogen interference in the *Aedes albopictus* HC line after 4 years of monitoring (Y. Wu, pers. com.). However, there is evidence of an impact of radiation on the density of *Wolbachia* in tsetse **(Demirbas-Uzel *et al.*, 2018)**. On the contrary, it did not modify *Wolbachia* density or pathogen interference in the *Aedes albopictus* line ”HC”**(Li *et al.*, 2021)**. Moreover, there is evidence that wMel density in the adults of the *Ae. aegypti* line declined when eggs were held at 26-36°C or above with complete loss at 30-40°C **(Ross *et al.*, 2019)** and no pathogen interference against Zika & Dengue 2 was observed in the *Ae. aegypti* line used in Singapore (C.H. Tan, pers. Com.). There is therefore a need to better characterize the impact of irradiation and mass-rearing on pathogen interference and CI conferred by *Wolbachia*.

## 2. Quality Control

Quality control methods for mosquito SIT

The term “Quality Control” (QC) in insect mass-rearing refers to the methods to assess and ensure the ability of produced sterile males to compete successfully with wild conspecifics to mate fertile females **(Boller *et al.*, 1981)**. The main objective of quality measurement is to detect any significant change in the produced insects, providing a rapid diagnosis so that the production process can be amended and ensure that sterile males will perform successfully after release.

Quality control in a mass-rearing facility needs to be divided into three basic processes: 1) quality control of the production, 2) quality control of the process and 3) quality control of the product. It is thus necessary to determine the correct size of the samples of eggs, larvae, pupae and adults, as well the number of repetitions (FAO/IAEA/USDA, 2014). The mass-rearing process must be monitored continuously to verify that each development stage meets the established requirements **(Calkins and Parker, 2005; Hernandez *et al.*, 2010**, see Figure 1).

As mosquitoes are maintained in artificial rearing conditions and are submitted to strong selection during colonization to increase yields, behavioral modifications may produce some mating incompatibility between mass-reared males and wild females **(Rull, Brunel and Mendez. M.E, 2005)**.

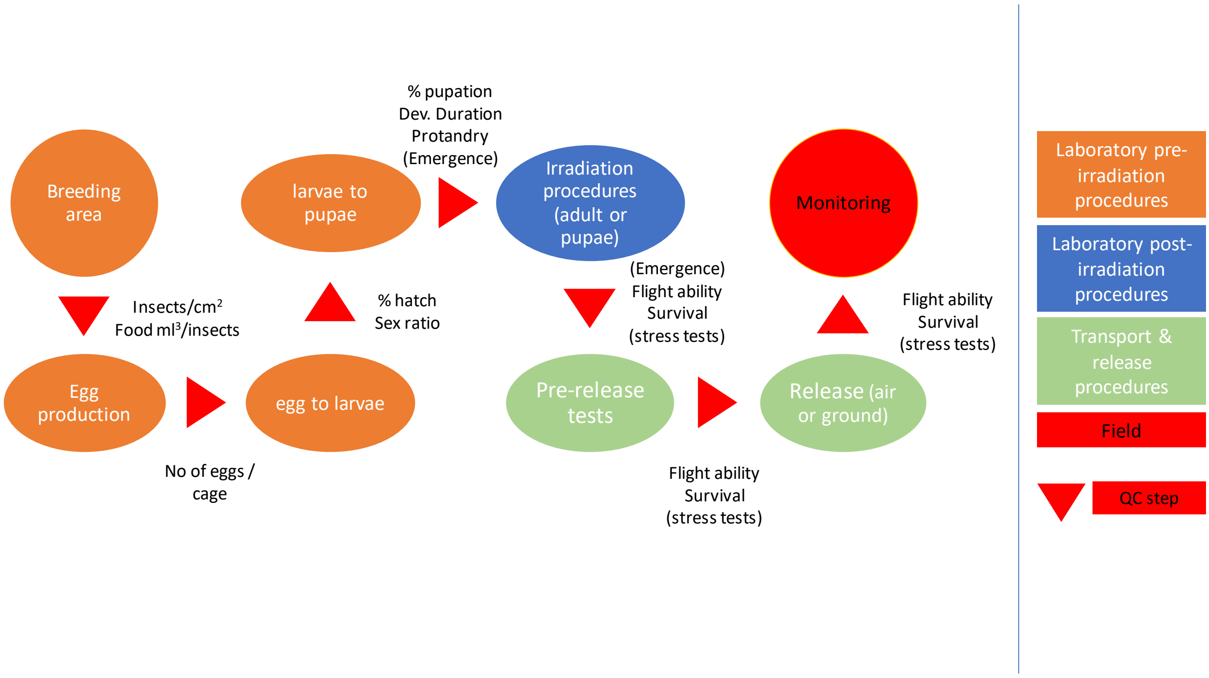



Fig. 1 Examples of quality control monitoring along the production chain of sterile male mosquitoes (Adapted from (FAO/IAEA/USDA, 2014))

Several quality-control tests have been developed and are regularly used along the production-release process **(Balestrino *et al.*, 2017; Culbert *et al.*, 2018**; **FAO/IAEA/USDA. 2014**). Most of these methods identified problems during rearing, irradiation, transport, holding, chilling, and release of the sterile males. Also, some field cage quality-control tests have been developed to evaluate male mating performance **(Rull *et al.*, 2012)**.
The procedures for releasing sterile males will generally be performed using the chilled adult technique, which immobilize the insects using low temperatures (6 to 10 ° C), which can also have a significant effect on the quality of sterile insects. For this reason, every batch of sterile males need to be monitored at each step of the production process, in order to detect any problem related with the final quality of the released insects **(Arredondo *et al.*, 2016)**.
Moreover, some more complex tests which are costly and time consuming need to be conducted at regular intervals, e.g. competitiveness tests in semi-field settings (Bellini et al. 2013).

## 3. Inter-lab studies

**1. Irradiation protocol including establishment of dose-response curves**
A survey organized between MSs revealed that the irradiation doses necessary to induce 99% sterility were very variable between sites. However, many variable factors can explain this result of which most can be controlled for (apart from the dose rate). It would be useful to organize an inter-lab study with a strict protocol to be followed, in order to ensure the comparability of the results (see Appendix I of the Irradiation guideline at://www.iaea.org/sites/default/files/2020-guidelines-for-irradiation.pdf).

A preliminary step will be to organise a cross-lab study to evaluate the uncertainties of existent dosimetry systems to verify doses that are applied in the different labs/ projects. The protocol for this dosimetry trial is presented in annex 4.

**2. Comparison of flight ability and semi-field competitiveness**

Flight ability has been demonstrated to be a sensitive proxy of male quality, by comparing it to other QC reference parameters, namely survival and mating compatibility. However, there is no information on its predictive value for male competitiveness.

An inter-lab study will thus be organized within this CRP to measure the correlation between flight ability and semi-field competitiveness, following the protocols presented in annex 5.

## 4. Logical Framework

**Logical Framework (table):**

| Narrative Summary | *Objective Verifiable Indicators* | *Means of Verification* | *Important Assumptions* |
|---|---|---|---|
| ***Overall Objective*** **develop and evaluate irradiation and quality control procedures to be used for sterile insect technique (SIT) applications, as part of AW-IPM programmes, to control populations of mosquitoes, vectors of human diseases** | N/A | N/A | Requests by Member States in the area of mosquito control using the SIT are increasing. To transfer this nuclear technology to Member States, the availability of irradiation and quality control procedures at large scale is an essential precondition. Biological material is available. |

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| **Specific Objectives** | | | |
| **1. Understand the factors that affect sterilization by irradiation and downstream performance of the sterile male mosquitoes** | At least three specific factors affecting irradiation described | Reports and published papers. | Specific factors affecting irradiation can be identified. |
| **2. Design and validate irradiation and dosimetry protocols for large numbers of mosquitoes, appropriate for operational programmes** | Protocol for irradiation and dosimetry developed. | Reports and protocols. | Irradiating large numbers of mosquitoes without impacting their quality is possible. |
| 3. **Develop and validate standard product QC procedures for sterile male mosquitoes** | At least three QC tests developed and validated. | Reports and or published papers. | QC tests allowing fast and cheap evaluation can be developed and adopted by MSs. |

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| **Outcomes** | | | |
| **1. Major drivers of variation in the efficacy of sterilization by irradiation identified and integrated into MSs protocols** | Protocols adopted | Data collected | Protocols can be integrated into MS production systems. |
| **2. Guidelines for large-scale irradiation adopted and implemented** | Guidelines developed | Data collected | Guidelines can be adopted by MSs. |
| **3. Guidelines for QC adopted and implemented within operational SIT mosquito programmes** | Guidelines developed | Data collected | Guidelines can be adopted by MSs. |

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| **Outputs** | | | |
| **1. Data on the impact of endogenous factors on irradiation efficacy including physiological and cellular processes available** | At least two endogenous factors impacting irradiation efficacy identified | Reports and or published papers | Biological material is available. Methods are available or can be applied to mosquitoes. |
| **2. Data on the impact of exogenous factors on irradiation efficacy available characterized** | At least two exogenous factors impacting irradiation efficacy identified | Reports and or published papers | Biological material is available. Methods are available or can be developed |
| **3. Impact of the potential introduction of colony genotypes and irradiation induced mutations into targeted populations through residual fertility quantified** | Frequency of transfer of mutations quantified | Reports and or published papers | Methods are available or can be developed |
| **4. Impact of irradiation on vectorial capacity including female behaviour, vector competence and longevity evaluated** | At least two traits related to vectorial capacity characterized | Reports and or published papers | Laboratory with appropriate level of biosafety for vector competence studies available. |
| **5. Impact of irradiation on cytoplasmic incompatibility and pathogen interference conferred by Wolbachia quantified** | At least two Wolbachia strains assessed | Reports and or published papers | Biological material available. Laboratory with appropriate level of biosafety for vector competence studies available. |
| **6. Irradiation and dosimetry procedures for SIT applications harmonized** | One procedure developed | Protocols published | MSs will adopt irradiation and dosimetry protocols |
| **7. Impact of irradiation relative to other production steps on the quality of male mosquitoes evaluated** | Impact of irradiation compared to at least two production steps (measure impact of production steps & irradiation on mosquito quality) | Reports and published papers | QC protocols are available or can be developed |
| **8. Quality control methods** | At least two quality control tests for the | Reports and published papers | QC protocols can be developed |

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| **to be applied to monitor the mosquito production process developed and validated**<br><br>**9. Quality control methods to be applied to monitor sterile male performance developed and validated**<br><br>**10. Results published in a peer reviewed journal** | production process validated<br><br>At least two quality control tests for sterile males performance validated<br><br>Papers drafted and submitted. | Reports and published papers<br><br>Journal issue with published scientific papers. | QC protocols can be developed<br><br>Data for publication available |

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| ***Activities*** | | | |
| **1. Announce project amongst established entomologists working on vectors and establish CRP** | Proposals evaluated and 10 Research Contracts, 10 Research Agreements and 1 Technical Contract awarded. | Signed contract and agreements. | Suitable proposals submitted, funding available and approval of Contract and Agreements by CCRA-NA committee. |
| **2. Organize first RCM to refine the logical framework and plan the overall activities of the CRP (3Q 2020)** | 1st RCM held 2021. | Participants' activities and logical framework revised. | Contracts and Agreements signed by counterpart organizations. |
| **3. Conduct Research and Development** | New knowledge created on irradiation and QC of mosquitoes | Scientific papers and reports from the participants | Methods and resources available. |
| **4. Organize second RCM to analyse progress in delivering research outputs and plan the next phase of the project (1Q, 2022).** | 2nd RCM held 2022. (Q3, in Vienna) | Participants and RCM Progress Reports. | Progress satisfactory. |
| **5. Organize a training on irradiation and quality control in conjunction with the second RCM** | Training held in 2022 (2-3 day workshop, before the 2.RCM) | Training report | Suitable location to held the training and training material available |
| **6. Conduct Research and Development** | New knowledge created on irradiation and QC of mosquitoes | Scientific papers and reports from the participants | Methods and resources available. |
| **7. Review the CRP after its third year** | Satisfactory progress of research agreements and technical contract | Report | Contracts and Agreements properly managed by counterpart organizations. Methods and resources available. |
| **8. Organize third RCM to analyse progress in delivering the research outputs and plan the final phase of the project. (3Q, 2023)** | 3rd RCM to be held 2023. | Participants and RCM Progress Reports. | Progress satisfactory and mid-CRP evaluation approved by CCRA-NA committee. |

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| **9. Conduct Research and Development** | New knowledge created on irradiation and QC of mosquitoes | Scientific papers and reports from the participants | Methods and resources available. |
| **10. Organize final RCM to assess the success of the CRP in reaching its objectives and review the final publication. (1Q, 2025)** | $4^{th}$ RCM to be held 2025. | Participants and RCM Final Reports | Final reports are submitted to the Agency. |
| **11. Evaluate the CRP and submit evaluation report.** | Satisfactory completion of research agreements and technical contract | Report | Contracts and Agreements properly managed by counterpart organizations. Methods and resources available. |
| **12. Publish the results of the CRP in a special issue of a peer reviewed journal.** | At least 20 publications accepted. | Scientific publications. | Consensus can be found on appropriate peer review journal and acceptance by journal obtained. |

## 5. References


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# ANNEX 1: List of participants

D44004-CR-1
First Research Coordination Meeting on Mosquito Irradiation, Sterilization and Quality Control
Seibersdorf, Austria
31 May to 4 June 2021

List of Participants
(as of 2021-05-06)

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## ANNEX 2: Agenda

**First FAO/IAEA Research Co-ordination Meeting**
**"Mosquito Irradiation, Sterilization and Quality Control"**
**May 31 – June 4, 2021**

Vienna, Austria – Virtual

**Monday May 31, 2021**

### Day 1: **The Americas (time zone)**

*Webex session open at 14:00 CET (Vienna time)*

CET (local time for presenter)

14.30 – 15.00 OPENING REMARKS

15.00 – 15:30 **Hanano Yamada & Hamidou Maiga**

*Irradiation studies & Quality Control methods and tools at the IPCL*

15.30 – 16.00 (10.30 – 11.00) **Jair Virginio** (Brazil, -5)

*Adult mosquito irradiation at Moscamed Brasil*

16.00 – 16.30 (10.00 – 11.30) **Dan Hahn** (USA, FL, -6)

*Thinking about hypoxia and temperature treatments for improving sterile male performance in the field.*

16.30 – 17.00 (10.30– 11.00) **Heath MacMillan** (Canada, (Ottawa) -6)

*Can we improve the field performance of SIT Aedes aegypti through assisted thermal acclimation?*

17.00 – 17.30 (10.00 – 10.30) **Patricio Ponce** (Ecuador, -7)

*Irradiation assays for Aedes aegypti from Ecuador*

17.30 – 18.00 (10.30 – 11.00) **Ariane Dor** (Mexico, -7)

*Development and evaluation of quality control methods for the application of the SIT in Aedes aegypti*

*BREAK 10 min*

1 hour Discussion

**Tuesday June 1, 2021**

Day 2: **Europe & Africa (time zones)**

*Webex session open at 12:00 CET (Vienna time)*

CET (local time for presenter)

12.45 – 13.00 OPENING REMARKS

13.00 – 13.30 (15.00 – 15.30) **Ambicadutt Bheecarry** (Mauritius, +2)

*Optimizing the quality of sterile Ae. albopictus males released as part of a Sterile Insect Technique (SIT) feasibility study in Mauritius*

13.30 – 14.00 (13.30 – 14.00) **Louis Clement Gouagna** (France)

*Standardization of irradiation process of Aedes albopictus males under mass-rearing conditions in support of SIT program in La Reunion Island*

14.00 – 14.30 (15.00 – 15.30) **Antonios Michailakis** (Greece, +1)

*Testing quality control methods for irradiated Aedes albopictus males*

14.30 – 15.00 **Florent Kuntz** (France)

*Dosimetry challenges and irradiation modality effect on mosquito*

15.00 – 15.30 **David Almenar** (Spain)

*Development of protocols and materials for medium-large scale sterilisation by ionizing irradiation and electron-beam technology, and Quality Control of Aedes mosquitoes*

15.30 – 16.00 **Romeo Bellini** (Italy)

*Scenarios for the application of SIT on Aedes invasive species in Europe*

*BREAK*

16.30 – 17.00 **Michael Samuel** (South Africa)

*Optimizing bulk irradiation of mass-reared Anopheles arabiensis males in prospective of a pilot sterile male release programme in South Africa*

17.00 – 17.30 (14.30 – 15.00) **Mame Thierno Bakhoum** (Senegal, -2)

*Quality control procedures of Aedes aegypti sterile males to ensure effective area-wide integrated management programmes*

17.30 – 18.00 (15.30 – 16.00) **Simon Sawadogo** (Burkina Faso, -2)

*Evaluating the influence of environmental factors on the swarming and mating competitiveness of sterile males of Anopheles arabiensis and the irradiation dosage on the vector competence of sterile females under semi-field and field conditions*

1 hour Discussion

## Wednesday June 2, 2021

## Day 3: Asia (time zones)

*Webex session open at 08:00 CET*

CET (local time for presenter)

08.45 – 09.00 OPENING REMARKS

09.00 – 09.30 (15.00 – 15.30) **Glenda Obra** (Philippines, +6)

*Irradiation, Sterilization and Quality Control of Dengue Mosquito, Aedes aegypti in the Philippines*

09.30 – 10.00 (15.30 – 16.00) **Yongjun Li** (China, +6).

*Quality control of long-term mass-reared Aedes albopictus for suppression*

10.00 – 10.30 (15.00 – 15.30) **Hadian Sasmita** (Indonesia, +5)

*Exploring the critical factors influencing the radiation sensitivity in irradiated males Aedes aegypti*

10.30 – 11.00 (15.30 – 16.00) ~~**Pattamaporn Kittayapong**~~ (Thailand, +5)

*Title to be provided*

11.00 – 11.30 (15.00 – 15.30) **Kajla Seheli** (Bangladesh, +4)

*Optimization of irradiation dose and quality assurance of sterile male Aedes aegypti: A laboratory study*

*BREAK*

1 hour Discussion

**Thursday, 3 June 2021**

Day 4**:**

Group discussions: (re)defining R&D workplan and goals: **open sessions** (*use the same link*)

**09.00-10.00** Vienna time: Matching & optimizing irradiator and dosimetry systems.
Discussion and Q&A time with Florent on getting our dosimetry right!

**10.00-12.00** Vienna time (see table on page 5 for your local time) Europe, Africa & Asia

**15:00-17.00** Vienna time (see table on page 5 for your local time) The Americas (& Europe)

Revision of the Logical matrix framework (to be edited on the document)

**ANY TIME** Other sub-group discussion, coordination & collaborations on specific activities, or One-on-one discussions on data/protocols/planned activities/etc (let me know by email if you wish to meet and when)

Final report- completion of all sections

**Friday, 4 June 2021**

Day 5**:**

**Open sessions**

**10.00-12.00** Vienna time (see table on page 5 for your local time) Europe, Africa & Asia

**15:00-17.00** Vienna time (see table on page 5 for your local time) The Americas (& Europe)

Final report compiled and completed

# ANNEX 3: Abstracts of presentations

## Optimizing the quality of sterile *Ae. albopictus* males released as part of a Sterile Insect Technique (SIT) feasibility study in Mauritius

**Ambicadutt Bheecarry, Diana P. Iyaloo, Khouaildi B. Elahee, Varina Ramdonee-Mosawa, Nabiihah R. Munglee, Nilesh Latchooman, Srutee Ramprosand, Surendra Puryag**

*Vector Biology and Control Division, Ministry of Health and Wellness, Mauritius*

When the national project MAR 5019 (aiming to investigate the use of the Sterile Insect Technique to control *Aedes albopictus* in Mauritius) was implemented; several areas for improvements were identified. Some of those areas are enumerated below and will be addressed during this CRP in Mauritius.

(1) Although the sterility of released males were assessed throughout MAR 5019, a quality control system to accurately monitor male fitness was lacking. Hence, building from the work started at the IPCL, male performance will be assessed in the laboratory, semi-field and field conditions and the results compared and evaluated with the aim of developing a quick and effective quality control system for sterile males.

(2) During irradiation studies conducted in a cesium-137 irradiator, the density of pupae at irradiation significantly impacted their level of sterility and pupae had to be irradiated at a density not exceeding 1500 per petri dish for an effective sterilization. Since gamma rays can only be effectively attenuated by very dense materials such as lead, it is unclear how pupal density could have impacted the level of radiation dose received. The main hypothesis is an increasing level of hypoxia with density, thus conferring increasing resistance to irradiation. This will be investigated during this CRP.

(3) In an operational sterile release programme, it is essential that a compaction system for sterile *Ae. albopictus* males be developed to ensure that males are transported in a practical, cost-effective way with reduced effects on their competitiveness. Preliminary works have started at the VBCD to develop optimized protocols for the irradiation of *Ae. albopictus* males in the pupal and adult stages and to compare male fitness using both protocols. Furthermore, the effect of dose-rate and energy of the rays on the sterility and performance of the males using two different type of gamma irradiators (Cobalt-60 and Cesium-137) will also be investigated.

(4) Finally, if travel restriction becomes more relaxed, the impact of irradiation on different strains of *Ae. albopictus* (originating from two dependencies of Mauritius), will be investigated.

# Quality control procedures of *Aedes aegypti* sterile males to ensure effective area-wide integrated management programmes

**M. T. Bakhoum[1], G. Diouf[1], A. G. Fall[1] and M.T. Seck[1]**

[1] Laboratoire National de l'Elevage et de Recherches Vétérinaires de l'Institut Sénégalais de Recherches Agricoles (LNERV-ISRA), BP 2057, Dakar, Sénégal

Although the sterile insect technique (SIT) remains the most effective strategy used to control insect pests, its application in area-wide integrated pest management (AW-IPM) programmes continues to increase in response to requests from the Member States. These requests include the development and refinement of the SIT packages for programmes to control mosquito populations. The successful implementation of area-wide integrated pest management, with an SIT component against these vectors, depends on the efficiency of irradiated sterile males to induce sterility in the target populations. Many factors from the sterile male production of mosquitoes to their release in the field determine the quality of released males and ultimately affect their performance in the field. The challenge is thus to perform adequate quality control procedures to monitor the quality of the sterile males of mosquito along the production chain and release, to ensure the effectiveness of implementing area-wide integrated pest management (AW-IPM) programmes. The main objective is to develop and evaluate adequate quality control procedures to be used for sterile insect technique (SIT) applications to control populations of *Aedes aegypti*. In this project, based in Senegal, we propose to assess the impact of the irradiation on the evolutionary response of *Aedes aegypti* populations in testing the flight ability and the competitiveness of *Aedes aegypti* males irradiated in the different stages (adult and pupae) under semi-field cages and field trial, including their survival and dispersal.

For the next 18 months, we will (1) assess the impact of dose responses in *Aedes aegypti* males irradiated as pupae and adult on the flight ability of irradiated males, and (2) evaluate the competitiveness of *Aedes aegypti* males irradiated in adult and pupae stages using doses giving induced sterility (IS) >99% under semi-field conditions.

## Testing quality control methods for irradiated *Aedes albopictus* males

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The evaluation of this sterile insect technique (SIT) for Greece has already begun since the autumn 2018 in the area of Vravrona (Municipality of Markopoulo) against the *Aedes albopictus.* For the SIT trials irradiated males are transported from the CAA facility (Italy) to the Benaki Phytopathological Institute in Athens by air express carrier. In order to conduct the SIT pilot program, adults were sent every week in chilled foam boxes. Under the frame of the current CRP program (CRP code: D44004), we assessed several experimental procedures trying to investigate different parameters that might indicate the quality of the transported sterile males. Initially, the residual fertility of Greek *Aedes albopictus* strain (Vravrona colony) was evaluated and compared with other European strains. The quality characteristics of the sterile males of *Ae. albopictus* Vravrona colony in comparison with those of the wildish males of a recently established laboratory colony. Specifically, we evaluated the effect of mating, food and water stress on male survival, Effect of mating on male longevity and the male mating performance (mating propensity and male mating competitiveness). The results showed that the quality importance of the transported sterile males was very good. As a next step we are planning to implement a Mark-Release-Recapture studies on the dispersal capacity and post-capture longevity of irradiated of the *Ae. albopictus*. We will also try to include in MRR studies non-irradiated males of the Asian tiger mosquito aiming to record the (remaining) lifespan of the recaptured individuals maintained under laboratory conditions to clarify how their exposure to natural settings shape their longevity.

## Standardization of irradiation process of *Aedes albopictus* males under mass-rearing conditions in support of SIT program in La Reunion Island

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Abstract. Under the phase 2 of the R&D program to study the feasibility of the Sterile Insect Technique (SIT) for the control of *Aedes albopictus* on Reunion Island, one of the main challenge is to upscale the mass rearing as well as to optimize the irradiation system for large number of mosquitoes. The factors influencing the sterility level of male *Aedes albopictus* exposed to ionising irradiation are complex and include both endogenous parameters, i.e. developmental stages, duration of rearing in the lab, geographic origin of mosquito strains, and exogenous factors such as the bulk number of mosquitoes to be irradiated, holding and exposure conditions. Early experiments suggested that 35-40Gy from gamma and X-ray sources is the most appropriate dose for implementing SIT for the control of *Aedes albopictus* on the Island. This presentation will provide an update of further radio-sterilization studies carried out in an ongoing SIT feasibility program, where we used varying number of *Aedes albopictus* male pupae (c.a. 2000 – 14000 pupae per irradiation cycle) derived from a mass-rearing system, and test different conditioning atmospheres in order to establish optimal conditions for the mass-production of sterile males, beside reducing the level of residual fertility and maintaining sterile male quality. Though our objectives have been to identify the dose of X-ray radiation and irradiation conditions that would fully sterilize male pupae under mass-rearing conditions, the accidental release of sub-sterile male mosquitoes and residual females in an SIT program is possible, and likely has some associated potential ecological and health risks. Further studies are planned (1) to examine the fitness of male and female offspring originating from sub-sterile male parents x fertile female crosses, (2) to document the effects of releasing semi-sterile irradiated male *Aedes albopictus* on the target population gene pool, and (3) to investigate the response of sterile female *Aedes albopictus* to arbovirus infection. The implication of such studies for mass release of sterile mosquitoes in SIT program will be discussed.

# Exploring the critical factors influencing the radiation sensitivity in irradiated males *Aedes aegypti*

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Several factors within the radiation sterilization process are linked to the radiation sensitivity of males *Aedes aegypti*. Those factors could come from the environmental conditions and physics during the irradiation process, such as atmosphere, dose rate, and temperature; and/or biological conditions including stage, age, size & weight, diapause, and nutritional state. Exploring those factors is critically important to improve or, at least, to maintain the quality of irradiated males, leveling their competitors in the field. The studies exploring the listed factors should receive more attention to develop consistent, reproducible, and reliable irradiation procedures as one of determining factors in successful SIT application against *Ae. aegypti*. Among the above-mentioned factors, we have been investigating life stage, age and temperature factors on irradiation process conducted in Gamma-cell 220 applying varied doses. Biological parameters related to males' quality have been characterized for each dose and factor. Briefly, males' longevity, induced sterility level and mating competitiveness index were affected by dose received, life stage and age. Higher temperature applied prior to the irradiation resulted in higher induced sterility level and lower longevity. Further works within the scopes of CRP will be investigating acute and chronic irradiation using different type of gamma irradiators; different levels of oxygen atmosphere before and during irradiation; and different origins of the species. In order to do those works, we have been preparing the two irradiators with appropriate dosimetry system; mastering the technique to create hypoxia and normoxia conditions; as well as collecting *Ae. aegypti* field strains from the different origins of Indonesia.

## Simultaneous application of MAT and SIT for management of B*actrocera dorsalis* in South Africa: environmental and physiological considerations

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Males of many *Bactrocera* Macquart (Diptera: Tephritidae) species are attracted to one of a number of semiochemical compounds referred to as phenylpropanoids. Due to their attractiveness, some of these chemicals, including methyl eugenol, are highly effective lures in traps that are also used for "male annihilation technique" (MAT). MAT can be used prior to sterile insect technique (SIT) programmes targeting *Bactrocera* species to reduce the abundance of wild males, thereby improving the sterile:wild male ratio. This sequential application of MAT followed by SIT was believed necessary to prevent attraction of released sterile males to MAT baits. However, it has been proposed that MAT and SIT may be applied simultaneously because prior exposure to a phenylpropanoid reduces subsequent male attraction to the same or a different chemical. An issue that needs to be addressed is whether pre-release nutritional treatments supported by joint FAO/IAEA-funded research are also compatible with simultaneous application of MAT and SIT. This is because sterile male *Bactrocera* fed yeast hydrolysate to improve their survival and mating performance respond more strongly to phenylpropanoid lures. This project, based in South Africa, will investigate some of the environmental and physiological variables that may interact with semiochemical pre-treatment to influence response of *Bactrocera dorsalis* (Hendel) to methyl eugenol. Specifically, we will: (1) Identify alternative, affordable and readily available semiochemicals for pre-release treatment of *B. dorsalis* that reduce responsiveness to traps baited with methyl eugenol; (2) Establish the responsiveness of *B. dorsalis* to traps baited with methyl eugenol when pre-treated with selected semiochemicals and nutritional supplements under varying semi-field conditions; (3) Determine whether pre-release treatment of *B. dorsalis* with selected semiochemicals and nutritional supplements affects flight, dispersal and metabolic rate; and (4) Document the field response to methyl eugenol by sterile *B. dorsalis* receiving pre-release treatments relative to untreated and wild males.

## Development and evaluation of quality control methods for the application of the SIT in *Aedes aegypti*

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In southern Mexico, a pilot project on *Aedes aegypti* Sterile Insect Technique is currently performed with a Genetically Diverse Strain (GDS). Initially, mosquitoes were chilled and manually released by ground; after that, they were released by air using a drone, involving an additional chilling step in the process. Because of the COVID-19 pandemic, releases have been stopped since March 2020, as well as the production of the mosquito's strain, making impossible to achieve any planned research. Nevertheless, in 2021, part of the specific objective 1 (to establish the baseline of the quality parameters of the mass-reared GDS) and the whole objective 3 (to assess the sexual performance of the mass-reared males) are attended. We are testing whether the chilling processes affects sterile males' survival, flight ability and sexual competitiveness. First, we will determine the effect of different chilling times on the survival and flying abilities of male mosquitoes under laboratory conditions. For that, four treatments plus a control (mosquitoes without chilling) will be performed. The first treatment consists of the manual release protocol, that is a chilling at 4 ° C for 25 minutes. The second, third and fourth treatment correspond to the air release protocol, consisting of a second chilling for 25, 50 or 100 minutes. For the survival test, the daily mortality of 100 males from each treatment will be evaluated until the last adult dies (three repetitions/treatment and control). For the flight ability test, 50 mosquitoes will be tested in a flight device for 2 hours (ten repetitions/treatment and control). To test the sexual competitiveness of irradiated males, the irradiated males will be chilled under two treatments: ground (on chilling at 4°C for 25 min) and air release (two chillings at 4ºC for 25 minutes each). The control will consist of unchilled irradiated mosquitoes. The experiment will be carried out according to the protocol established by the IAEA and the Fried (1971) competitiveness index will be calculated.

## Optimization of irradiation dose and quality assurance of sterile male *Aedes aegypti*: A laboratory study

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A country of some 160 million people, Bangladesh had struggled against its worst outbreak of dengue fever causing 164 deaths in the year 2019. Just two years back in 2017 chikungunya, another *Aedes* borne disease created significant concern in the country as more than 13000 people were infected. When it comes to transmitting diseases among people, mosquitoes are unsurpassed in the economic and health burdens they impose. In the absence of efficient drugs or vaccines and given the need to reduce the use of insecticides, international efforts are required to develop and implement new, complementary control techniques for mosquito species. The nuclear Sterile Insect Technique (SIT) is one such technique. Therefore, logical and phase conditional approach for mosquito population management through SIT could be considered very promising for future dengue mosquito management programme in Bangladesh. Insect Biotechnology Division of Institute of Food and Radiation Biology under the IAEA CRP grants, previously had established a mosquito insectary and has accomplished some basic researches on mass rearing, male sterility dose, adult male longevity and egg hatching rate etc. As radiation is deleterious to mosquito, careful studies are needed to select an optimal irradiation dose that will ensure the quality of sterile males for field release. Hence, currently we are focusing to determine the appropriate radiation dose that may satisfy the fitness qualities of irradiated male mosquito of *Aedes aegypti* for SIT applications. This study was conducted in laboratory conditions. We determined the effects of different doses of gamma radiation on flight ability, adult longevity and wing size of sterile males. The work plan for next two years are as follows: 1) Mating competiveness analysis of the irradiated males of *A. aegypti*. 2) Sex separation process will be further improved. 3) Up-scaling the production of sterile *A. aegypti* males up to 50 thousand per week. 4) The irradiation process for large volume of male pupae will be established.

## Quality control of long-term mass-reared *Aedes albopictus* for suppression field trial in China

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The endosymbiotic bacterium *Wolbachia* is being developed as a tool to suppress mosquito populations and their transmitted pathogens, with successful field trials in multiple countries having resulted in efforts to scale-up the capacity to mass-produce mosquitoes for release. However, major challenges exist to achieving this goal, including concerns that mass-reared mosquitoes will adapt to laboratory conditions during long-term maintenance and experience inbreeding depression, resulting in poor performance of the released mosquitoes in the field. Here, we assessed the performance of the *Aedes albopictus* HC line infected with a triple-strain *Wolbachia* after mass-rearing at scaled-up densities of up to 15 million mosquitoes per week for over 50 generations. In comparison to the wild-type GUA line, the HC mosquitoes had desirable characteristics for mass-rearing and release, including robust male mating competitiveness, high female reproductive capacity, reduced vector competence for dengue virus, and increased *Wolbachia* density. Although the larval survival rate of the HC and GUA lines was similar, the HC larvae developed significantly faster, possibly because of up-regulation of the molting hormone 20-hydroxyecdysone-related gene *E75* in the HC larvae. Our results indicate that over many generations mass-reared mosquito lines can retain their quality if large effective population sizes with sufficient genetic heterogeneity are maintained under optimized rearing conditions, and demonstrate the long-term feasibility of deploying *Wolbachia*-based approaches for area-wide management of mosquito vectors for disease control.

## Irradiation assays for *Aedes aegypti* from Ecuador

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Dengue, zika and chikungunya diseases are present in Ecuador and affect mainly the population on the Pacific coastal zones, where dengue is considered endemic. Every year there are thousands of confirmed dengue cases. Chikungunya and Zika cases, since the first locally acquired cases reached high incidences in the coastal provinces and in the Amazon basin region.
The National Institute of Research in Public Health (INSPI) with IAEA support engaged in the SIT (Sterile Insect Technique) program to control *Aedes aegypti* populations in continental Ecuador and the Galapagos Islands.
We are monitoring the population of *Aedes aegypti* in the continent (Lita and Cachaco) and the Galapagos Islands. A colony of the insect from Lita has been reared in the laboratory to implement the SIT essays.
A critical part of the technique is to determine the radiation doses to sterilize the insects maintaining the competitiveness of males to mate with wild females when released in the field.
We have started a study to determine the doses to sterilize male mosquito pupae and measure the effects on mosquito male performance.
The irradiation is done in a semiautomatic irradiator JLShepard, Model 109 Cobalt-60, with 157.75 Gy /y dose rate. A dose map of the canister was done using Alanine dosimeters (Bruker, pellet type) to obtain dose rate and the calibration curve.
A canister was designed to hold seven plastic containers (30 ml) in five levels. Three pupae were placed in each container, 21 for each dose. The doses used were 40, 55, 60, 70 and 80 Gy. Radiation time varies from 14 (40 Gy) to 28 minutes (80 Gy). Preliminary results show high mortality rates (pupae and adults) as radiation doses increase. Mortality in the control group was 19%, with 40 Gy 38.1%, 55 Gy 61.9%, 60 Gy 57.1, 70 Gy 85.7%, 80 Gy 81%). Fertility rates (mean number of eggs per non-irradiated female) varied from 4 (control), 11 (40 Gy), 0 (55 Gy), 5 (55 Gy) 4 (60 Gy), and 0 (80 Gy). Viable eggs from irradiated males x non-irradiated females were produced only at 40 Gy (20%).
The next 18 months we plan to increase the number of irradiated pupae and measure the effects of radiation in the laboratory and field cages.

## Optimizing bulk irradiation of mass-reared *Anopheles arabiensis* males in prospective of a pilot sterile male release programme in South Africa

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*Anopheles arabiensis* is a dominant mosquito vector of malaria in sub-Saharan Africa and the primary vector of the disease in South Africa. While the country has made great strides in malaria control through the use of indoor residual spraying of insecticide – namely DDT, supplemental measures are necessary to achieve the target of eliminating local transmission of the disease by 2023. The sterile insect technique (SIT) has shown potential to suppress and possibly eliminate target populations of non-vector pest species as well as *Aedes* arbovirus vector species. As such, it may be a feasible option for the country's vector control arsenal, as well as have implications for other sub-Saharan African countries aiming to control this malaria vector.

To date, we have collected several years of surveillance data at a target site in Mamfene, KwaZulu Natal and have established a wild type strain of the predominant *An. arabiensis* vector at the NICD, Johannesburg. A genetic sexing strain was crossed with the wild strain to provide us with a suitable strain for mass rearing, designated as GMK. We have also investigated alternative sexing methods, and produced irradiation data as a platform for the prospective bulk irradiation – including dosimetry. A customized irradiation canister was also designed to contain mosquito pupae for baseline irradiation investigation. Finally, we have a newly established mass rearing facility where we are currently upsizing the colony for large scale experiments, including a pilot release of sterile males planned for 2021. This follows multiple mark-release-recapture exercises conducted between 2016-2018.

Our aim in the immediate future is to optimize a process for the bulk irradiation of *An. arabiensis* mosquitoes and to develop appropriate quality measures. This will primarily require further investigation into a suitable irradiation surface/canister for large quantities of mosquitoes and assessment of the impacts of pupal density, the absence and presence of water, and, the absence and presence of oxygen on sterilization and fitness. We will also investigate the potential for irradiating adults of the species and develop a standardized method for conducting this, if feasible. The current target is to consistently be able to produce 50,000 sterile male mosquitoes per week by the end of 2021 and henceforth expand capacity and production.

## Irradiation, Sterilization and Quality Control of Dengue Mosquito, *Aedes aegypti* in the Philippines

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The implementation of this CRP Project started only in the last quarter of 2020. For the first year, the objective was to determine the sterilizing dose for *Aedes aegypti* and determine the effect of irradiation on survival and mating of Ae. *aegypti*. Dose mapping was initially done using alanine dosimeters to determine the position of the minimum and maximum dose absorbed by the sample using the container designed for the test. Irradiation of male *Ae. aegypti* pupae was done using the new PNRI Gamma Irradiator (Ob-Servo Sanguis) procured through the IAEA TC project PHI5033, which became operational June 2020. Preliminary trials included only two doses (15 and 90 Gy) and a control lot. Survival did not differ significantly between 15 and 90 Gy, including the control. For the subsequent trials, pupae were irradiated using six doses ranging from 15 – 90 Gy at 100% shielding. Unirradiated pupae served as the control. The longevity of adult males, in general, decreased with an increase in dose. Based on the results of the sterility tests, fertile eggs were observed even at the highest dose (90 Gy) in Trial 1, but not in Trials 2 and 3, where complete sterility was observed at 75 Gy. For the next 18 months, we will be continuing with the maintenance of our stock colony, radiosensitivity studies using 50 and 25%shielding, and quality control of irradiated and control *Ae. aegypti.* We will also try to test on supplements for irradiated mosquitoes. If the situation will permit, we will visit another potential site for SIT.

# Development of protocols and materials for medium-large scale sterilisation by ionizing irradiation and electron-beam technology, and Quality Control of Aedes mosquitoes

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The Sterile Insect Technique (SIT) has proven to be effective to suppress the wild mosquito populations at a small scale. However, the actual feasibility of SIT as a valid area-wide vector control method has to be tested at large scales. Most of the methods currently used in pilot projects will not be directly applicable to large scales considering a cost-effectiveness point of view. The Spanish public company TRAGSA is currently involved in the research of large-scale methods in Aedes SIT. This research includes the mass sterilization of males and the application of quality control (QC) systems for the mass production of mosquitoes.
Our recent experience in the pilot project against *A.albopictus* combined with new research in the area of sterilization by ionizing radiation in the genus Aedes shows that there is an unexpected inconsistence concerning the outcome of sterilization, even when similar methodologies are in use. The identification of the key factors involved in this lack of uniformity is currently a subject of major importance for the Aedes-SIT pilot projects. Although traditionally the sterilization at pupal stage has been preferred in SIT projects, there is an increasing interest in the sterilization in adult stage, since there may be advantages that can exceed the inconveniences of their difficult handling. On the other hand, the electron beam technology is currently used for the sterilization of millions of Medflies in Valencia. Our goal is the development of protocols for the sterilization of male mosquitoes with beam electron systems. We will explore the possibility of the irradiation in pupae and adult stages. Concerning QC, our goal is the incorporation of quality control tests in a global quality system that includes the routine performance of quality tests and an integrated system of quality information management. This protocol of QC will also include the routine irradiation dosimetry.
For the next 18 months we plan to (1) Analyse the collected data on sterilization during the pilot project; (2) Analyse the data on QC during the pilot project and stablishment of QC parameters and thresholds; (3) Research on the factors associated to heterogeneity in the induced sterility to pupae with Gammacell 220, including geometry of dose, environment, and pupae condition; (4) Validation of containers specifically designed for the irradiation and transport of pupae and adults in Gammacell 220 and electron-beam (5) Research on the sterilization by electron-beam; (6) Development of protocols for the routine dosimetry for Gammacell 220; (7) Evaluation and research on the application of image analysis to CQ methods.

# Evaluating the influence of environmental factors on the swarming and mating competitiveness of sterile males of *Anopheles arabiensis* and the irradiation dosage on the vector competence of sterile females under semi-field and field conditions

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Due to the insecticide resistance developed by several malaria vector species and also to the lack of compliance of malaria control tools in the community level, the only use of insecticide- based control tools become challenging to reach the elimination level of malaria in the endemic African countries. The development of complementary methods became a crucial need. Among them the use of biological or genetic control of vectors being developed in lab level began promising. However, prior to implementing such tools or strategy in public health level, many steps remain to be rabbeted. In this perspective IAEA supports all countries aiming to develop biological control of both arboviruses or malaria vectors using the release of sterile males by irradiation alone or in combination with Wolbachia. But to better implement these techniques basic studies are needed to refine, standardise and sharing protocols. The mean objective of this study through this CRP is to test the competitiveness and the vector capacity and competence of respectively irradiated males and females of *An. arabiensis* under the influence of external factors such as temperature, seasonality, irradiation dosage. Specifically, we'll work on: (1) the kinetic of swarming and mating patterns of irradiated males in the lab using 3D system according to two irradiation dosages and temperature points, (2) the competitiveness of irradiated males in malaria sphere in dry and wet seasons (3) the density/dispersion and survival of marked/released and recaptured of irradiated males in the field around the river sides (4) the vector competence and capacity of irradiated females according two irradiation dosages.

## Thinking about hypoxia and temperature treatments for improving sterile male performance in the field.

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An important facet of sterile insect technique (SIT) is to consistently deliver high-quality, sexually competitive sterile males to the field that will be able to perform well. Yet, many steps in the sterile insect production and distribution pipeline can have negative effects on male performance: from mass rearing and handling to sterilization, shipping, and release protocols. Here I will give two case studies about how our lab and collaborators have used a variety of techniques in evolutionary physiology research to ameliorate the stresses incurred by insects at various stages in the sterile male pipeline. One important consideration for irradiation of large groups of insects is whether insects have access to adequate oxygen. Due to either accidental crowding or intentional packaging of insects in modified or controlled atmospheres, experiencing hypoxia prior to and during irradiation can affect both the dose needed to provide adequate sterility and post-irradiation male performance. I will discuss work in our lab that has shown irradiating *Aedes aegypti* pupae in low oxygen atmospheres increases the radiation dose needed to achieve 99% sterility and potentially improve post-irradiation male performance. Then, I will outline work currently underway that explores the effects of irradiation of compacted adult males under low-oxygen atmospheres on sterilizing doses and post-irradiation male performance. A second important consideration for sterile insect programs is to begin releases early in the season before wild populations grow large enough to be difficult to affect. Yet, males raised at warm temperatures in the lab may not perform well at seasonally low temperatures in the field. I will discuss work from our lab and collaborators showing substantial changes in thermotolerances of wild field populations across an entire mosquito community in North Florida, data that suggest wild males may be better prepared to perform in cool springtime conditions than lab-reared males. Then, I will outline work in progress to characterize the timescales over which lab-reared *Ae. aegypti* adults may be acclimating under field conditions to try to improve sterile male performance.

## Can we improve the field performance of SIT *Aedes aegypti* through assisted thermal acclimation?

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The majority of insects, including *Aedes aegypti*, are chill susceptible, meaning they suffer from performance deficits, sustain injury, and die at temperatures well above those that cause them to freeze. *Ae. aegypti* have historically been restricted to tropical and subtropical regions, but adults have been recently and repeatedly found in more poleward climates. This suggests that adult *Ae. aegypti* are capable of overwintering, but very little is known about their capacity for, and plasticity of, thermal performance and whether/how this plasticity could be used to improve performance of sterile males released in the field when the climate is suboptimal for release. We are working to characterize how thermal environments influence male *Aedes* performance in the laboratory and identify the physiological mechanisms that underlie plasticity in thermal performance observed. Our early work on this topic confirmed that *Ae. aegypti* are indeed capable of cold acclimation that mitigates cold-induced injury. In the coming months, we will extend this understanding with a focus on how chilling specifically impairs reproduction in this species and whether and how specific thermal treatments can mitigate these effects. We plan to do this using a custom-built laboratory system capable of producing 10-20 dynamic thermal environments for different animals simultaneously. Ultimately, we hope to test promising pre-treatments in a field setting using SIT males in order to develop best practice recommendations in matching thermal pre-treatments of sterilized insects to the prevailing climate.

## Dosimetry challenges and irradiation modality effect on mosquito

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X-ray irradiation of pupae and larvae becomes more and more attractive compared to gamma irradiation due to environmental and economic reasons. However, the usage of low to medium energy X radiation (lower than 300 keV) for low dose applications such as SIT treatments implies to control this process with sensitive, well characterized, and calibrated dosimetry systems.

Dosimetry for this kind of radiation energy is not trivial and if Cobalt60 calibrated dosimeters are used, one may have up to 30 % deviation due to the radiation spectrum differences.

In the past years, Alanine/EPR dosimetry system's response have been evaluated at Aerial. The first objective of this project is to set up a protocol which helps estimating the X radiation energy spectrum characteristics of partner's X- ray generators.

Traceable primary dosimetry with ionization chambers needs to be setup for this purpose and will make it possible to calibrate the Alanine /EPR dosimetry system in this particular radiation field. This would ensure consistency of irradiation doses between this CRP partners whatever the radiation type and energy in use.

The second work package of the proposed project is dedicated to radiation modality and dose rate impact on irradiated larvae and pupae. Samples from partners will be irradiated at same dose with low (up to100 kV), medium and high (starting from 0.8 MV up to 7 MV) energy X-rays as well as medium (2.2 MeV) and high (10 MeV) energy electron beams.

Dose rates (Gy/min) will be selected to obtain at least 1 decade difference between experiments. Thus, after post irradiation characterizations performed by the partners, irradiation modality and dose rate effect will be assessed. (Selection of larvae and pupae type is left to the competent partners.)

# ANNEX 4: Intercomparison of dosimetry systems

## PROFICIENCY TESTING BY INTERCOMPARISON OF DOSIMETRY SYSTEMS IRRADIATED WITH GAMMA (COBALT 60 SOURCE), 10 MEV ELECTRONS OR HIGH ENERGY X RAYS

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**INTRODUCTION**

The Proficiency Testing Programs by interlaboratory comparisons have proven to be a useful tool to give opportunity for participants to demonstrate their technical competence and ensure the quality and traceability of their measurements. It may also help to identify problems (if any) related to the performance of personnel, equipment calibration and adequacy of methods.

This exercise is developed to evaluate two aspects:

Part 1: The ability of participants to meet preset dose values (targeted doses).

Part 2: The ability of the participants to measure doses actually applied.

**REFERENCES**


ISO/IEC 13528:2005, Statistical methods for use in proficiency testing by interlaboratory comparisons.

NPL Report CIRM 29 “Guidelines for the Calibration of Routine Dosimetry Systems for use in Radiation Processing”; September 2009

ISO 14470-11 Food irradiation -- Requirements for the development, validation and routine control of the process of irradiation using ionizing radiation for the treatment of food

ISO/ASTM 51204-12 Standard Practice or Dosimetry in Gamma Irradiation Facilities for Food Processing

ISO/ASTM 51431-11 Standard Practice for Dosimetry in Electron and Bremsstrahlung Irradiation Facilities for Food Processing

ISO/ASTM 51607:2013 “Practice for Use of an Alanine-EPR Dosimetry System”

ISO/ASTM51275-13 Standard Practice for Use of a Radiochromic Film Dosimetry System

ISO/ASTM51650-13 Standard Practice for Use of a Cellulose Triacetate Dosimetry System

ISO/ASTM 51276-12 “Practice for Use of a Polymethylmethacrylate Dosimetry System”

ISO/ASTM 51401-13 Standard Practice for Use of a Dichromate Dosimetry

ISO/ASTM 51702-13 Standard Practice for Dosimetry in Gamma Radiation Facilities for Radiation Processing.

**PROFICIENCY TESTING MANAGEMENT**

Testing dosimeters

Proficiency test dosimeters: 4 alanine dosimeters (containing 4 pellets each) for each participant (1 is identified as control (Control / Do not irradiate) provided by Aérial.
For each target dose value, only one provided alanine dosimeter and if necessary one dosimeter from the participant dosimetry system is irradiated.

Confidentiality

To ensure confidentiality performance of the participants, Aérial will safeguard the results issued by each participant so as to maintain the confidentiality of the data and results for the performance of participants.

Schedule

Date of reception of dosimeters by participants: October 2022
Deadline for reception of irradiated dosimeters by Aérial: December 2022
Date of report delivery: March 2022

Parameter to be evaluated

The parameter to determine is the delivered dose to the dosimeters and the ability to target a given dose.
Dosimeters should be irradiated to doses (to water) of 35 Gy, 70 Gy and 150 Gy.
At this stage, irradiation with gamma (Cobalt 60 source), 10 MeV electrons or with X Rays (1 MV accelerating voltage minimum) is requested.

Proficiency testing registration

For registration, each participant will be sent an invitation email. Individual acknowledgement for participation is required to get the dosimeters.
Aerial will send to the participants, via the IAEA IPCL a package containing:
a) 3 alanine dosimeters (each dosimeter contains 4 pellets)
b) 1 alanine dosimeter to preserve as control, which should not be irradiated.

Proficiency testing progress

After registration, Alanine dosimeters will be sent to each participant along with an irradiation form, which must be completed by the participant after irradiation and sent with rapid mail to Aerial along with the irradiated dosimeters at the following address:

**Florent KUNTZ**
**IAEA Intercomparison**
**Aerial-CRT**
**250 rue Laurent Fries**
**67412 Illkirch**
**France**
**@: florent.kuntz@aerial-crt.com**
**Phone: +33 3 88 19 15 17**

Each dosimeter referred to in paragraph a) should be irradiated only with one of the indicated doses, (35Gy, 70 Gy, and 150 Gy)
The control dosimeter referred to in paragraph b) must remain with the other 3 dosimeters mentioned in a), except at the time of irradiation. The control dosimeter should never be irradiated.
The minimum dosimeter temperature has to be measured at the start of irradiation process, using a thermometer.
The maximum dosimeter temperature during irradiation has to be measured/evaluated throughout the irradiation process, using a thermometer/GEX thermolabel/experience. (Alternatively, measure the temperature near the dosimeter before and after irradiation and calculate effective temperature).

For each dose point, only one alanine dosimeter described in paragraph a) should be used, plus routine dosimeters belonging to the participant dosimetry system.

To ensure electronic balance, alanine and participant´s dosimeters must be enclosed with polystyrene or PMMA of 3 to 5 mm thickness.

The design and manufacture of container phantom of dosimeters is in charge of the participant. It must ensure that Alanine dosimeter and routine dosimeter from participant gets the same dose. The dimensions of alanine dosimeters are found in Annex.
Measurements of dose from dosimeters belonging to the participant´s dosimetry system must be made using the technique and equipment used by the participant in their normal routine work.
Participants must return to Aerial the irradiated Alanine dosimeters and the control dosimeter, along with the irradiation form right after irradiation is completed.
Irradiation form should be completed with the results of the participant's dosimeters dose, temperature of alanine dosimeter before irradiation and maximum temperature during irradiation, irradiation date, irradiation duration, radiation type, participants details, ...

**STATISTICAL EVALUATION**

Performance evaluator

xi dose value of the dosimeter irradiated by the participant measured by Aerial
X dose value given by the participant (close to the assigned 35Gy, 70 Gy, and 150 Gy)
u(xi) uncertainty (k=1) on dose measured by Aerial
u(X) uncertainty (k=1) on dose given by participant

The evaluator will be the Z performance parameter:

$$Z = \frac{xi - X}{\sqrt{u(xi)^2 + u(X)^2}}$$

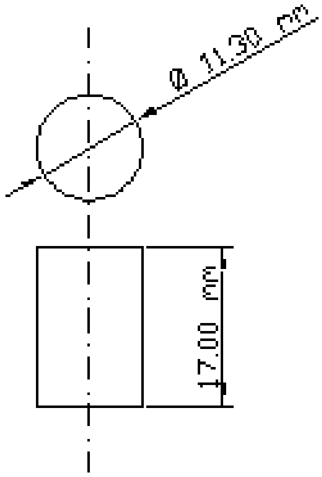
The acceptability criterion is:
If $-2 \leq Z \leq 2$ the result is considered acceptable.
If $\mathbf{2} < |z| < \mathbf{3}$ the result is considered questionable.
If $|z| \geq \mathbf{3}$ the result is not acceptable.

NOTE: The statistical treatment of the data may vary from that described according to the feedback of the participant's results.

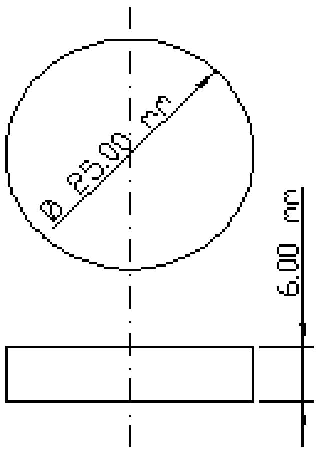
REPORT
Once the results are obtained from the participants, a preliminary report with the confidential results will be made and presented to IAEA.
Final results will be presented during the second RCM of the CRP 'Mosquito Irradiation, Sterilization and Quality Control'.

## ANNEX

### Dosimeter holder dimensions for gamma irradiation




**Dosimeter holder dimensions for electron beam irradiation**




| Aérial | IRRADIATION FORM | ID FE/I 01 - - - - / - - - - - - - |
|---|---|---|

**IRRADIATION FORM TO BE COMPLETED BY CRP PARTICIPANT**

| Participant details: | Contact person: |
|---|---|
| Full postal Address : | Phone :<br>Fax : |
| | E-mail : |

**Irradiation information**

| Dosimeter ID | Irradiation date | Irradiation duration | Temperature before irradiation ( °C ) | Maximum temperature during irradiation ( °C ) | Radiation type (XRay, γ, EBeam) | Energy (MeV) | Assigned dose (kGy) |
|---|---|---|---|---|---|---|---|

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| **Control** | **Do not irradiate** | | | | | | |

**Absorbed dose readings with participant's dosimetry system(s)**

| **Dosimeter ID** | **Participant dosimetry system** | **Measured dose (kGy)** | **Uncertainty (k=2) (kGy)** |
|---|---|---|---|
| | | | |
| | | | |
| | | | |

Date:

Visa:
Please return this form with irradiated dosimeters to:

Florent KUNTZ
IAEA Intercomparison
Aérial-crt
250 rue Laurent Fries
F 67412 Illkirch
France

E mail : florent.kuntz@aerial-crt.com

## ANNEX 5: Inter-lab comparison of flight ability and semi-field competitiveness

# How is male Aedes mosquito flight ability data related to semi-field competitiveness index?

Objective: To know whether flight ability scores are correlated to male mating competitiveness.
Biological material: Aedes aegypti and Aedes albopictus
Rearing, irradiation and experimental conditions:
Rear male mosquitoes following standard validated protocols,
Provide 10 % sucrose solution ad libitum to adults
Make sure that the rearing schedule gives males of desired and synchronized age groups

Pupae sampling: Aliquot 110 pupae/sex in 100mL- cups (males and females separate). Collect enough for each experiment and the number of replicates needed (see below for flight tests and competitiveness designs).

Adult-compaction: after emergence, chill adult cages (15×15×15cm) into the cold room (4°C for 10min) to knock down the mosquitoes; use a month aspirator to collect mosquitoes from each cage and transfer to 1×1×1 cm compaction boxes (100 males/cm$^3$). Close the box with a piece of netting (5×5cm) and a plastic rubber band (1.5cm=diameter) (Annex 2)

Irradiation: Irradiate en masse (100males/cm$^3$) using 1×1×1 cm compaction boxes for adults (1-2 day-old) or pupae (24h-30h-old) following standard irradiation procedures (See [Guidelines for Irradiation of Mosquito Pupae in Sterile Insect Technique Programmes | IAEA]
, Appendix I and III) with the dose inducing 99% sterility for your strain/irradiator. A higher dose will be used (40 Gy more for the effect of high dose irradiation on mating competitiveness experiment).

Laboratory room test conditions for flight test: 26 ±2 C, RH%= 70±10, light: 500-1000 lux; time= 8-10 am (morning).

Semi-field large cage size: 175×175×175 cm, 5.36 m$^3$ (Live Monarch. Boca Raton. USA)

## Experiment. Effect of high irradiation dose

Irradiation: Males of 2-3 day-old will be irradiated with low irradiation (inducing more than 99% sterility) and high irradiation (40Gy more than the low dose) doses.

A_ Flight ability
Three treatments (low, high doses and non-irradiated)

Run the flight test on irradiated (with low and high doses) and non-irradiated male mosquitoes
Three replicates for each treatment

B_ Competitiveness in large cages
The following design will be used for the competitiveness. The test will be performed 1 day after irradiation with 3-4-day-old males.

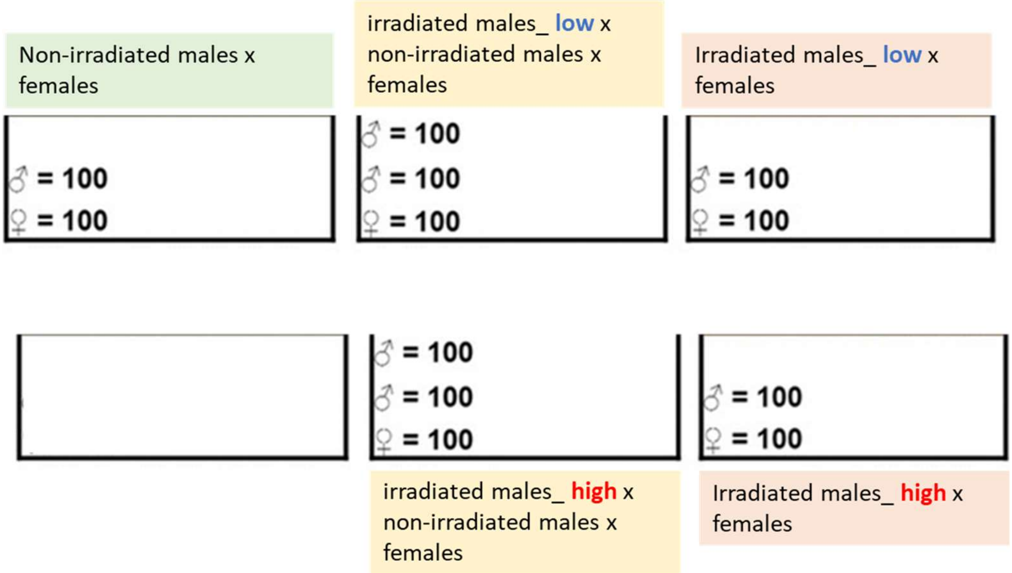



Figure 1. Experimental design in semi-field conditions. Three control cages (irradiated (low, high doses) and non-irradiated males only) and competition cages, each containing 100 males (irradiated and/or non-irradiated) and 100 virgin females. Low and high stand for low irradiation (inducing more than 99% sterility) and high irradiation dose (40Gy more that the low dose)

Procedures:
Only competition cages will be used in large cages in the semi-field settings
Control mating cages will be 30 × 30 × 30cm but placed in the semi-field conditions
Three cages for each competition treatment (6 large cages in total) will be placed in semi-field conditions
Males (irradiated and non-irradiated) will be released first and will be allowed to acclimate for 30min prior to releasing females (2-3-day-old).
After the mating period of 24 hours, females will be collected using mechanical aspirators, chilled to remove potential remaining males and cages.
Females will be brought back to the laboratory
Collected females' numbers will be recorded.
Females will be offered a blood meal for 2 consecutive days (30-60min/feeding event).
Daily survival will be monitored and egg cups for egg collection offered. One egg batch will be collected.
Competitiveness index will be calculated following Fried formula (see below parameters to be measured)
All females will be dissected to assess insemination rates (number of spermathecae filled with sperm) after egg collection.
PS: the use of rhodamine will allow true competitiveness

## Parameters to be measured:

Flight test:
Number of escaped (flyers) and non-escaped (non-flyers) males
Competitiveness:
Number of eggs per cage (based on females recaptured from semi-field cages)
Fecundity for each treatment by dividing the number of eggs laid by the number of females still alive (before egg collection)
Insemination rates to estimate the average number of females that laid eggs and the average number of eggs laid per female; to assess mating propensity/treatment
Egg hatch rates (fertility) by dividing the number of hatched eggs counted by the number of laid eggs (hatched + non-hatched). Eggs will be dried (1 week) prior to hatching (See Guidelines for Irradiation of Mosquito Pupae in Sterile Insect Technique Programmes | IAEA, pages 20-24)).
The competitiveness index (C) (Fried index): using egg hatch rates from the non-irradiated control (Ha), irradiated control (Hs) and competitiveness treatments (Ho) as follows: $C = ((Ha–Ho)/(Ho–Hs)) \times (N/S)$; where N is the number of non-irradiated males and S the number of irradiated males.

Each experiment will be repeated three times.

Script of the Flight ability test video
Link to the video:
https://www.dropbox.com/s/22tks0hhp31qokq/Flight%20test%20device%20instructional%20video%20cut2%20with%20sub%20and%20music.mp4?dl=0

Script of the Flight ability test video:
0:00-0:06 "The flight ability of mosquitoes can be measured using a Flight Test Device, as developed by the Insect Pest Control Subprogramme."
0:19-0:28 "The Flight Test Device consists of six separate parts:
- a base plate;
- a transparent containment box with an open top and a circular netted opening;
- a top cover;
- the flight tube itself, composed of 40 individual tube's each 25 cm high with an inside diameter of 8 mm placed inside a large containment tube;
- a 12V fan with an air flow of 0.218$m^3$/min, am acoustic noise of 20.6dB and a rated speed of 6000rpm;
- and finally, a rubber base ring with a square of netting."

0:29-0:39 "To assemble the Flight Test Device, begin by placing the base plate inside the rectangular containment box. The base should be placed with the metal rods facing downwards so that there is a space between the bottom of the containment box and the base plate.
0:39-0:51 "Next, cover the rubber base ring with the square of netting and fit it to the bottom of the large containment tube. Make sure the rubber ring fits the containment tube tightly, but do not to cover the small, 1 cm hole at the bottom of the tube yet."
0:52-0:57 "Move the tube inside the rectangular containment box and cover the latter with the top cover."
0:59-1:06 "Put 2-3 small pellets of BG lure from Biogents, Regensburg, Germany in the decoy cap on the top cover."
1:07-1:13 "Place the 12V fan directly on the decoy cap facing downwards so that air is blown into the containment box."
1:17-1:24 "Switch on the fan for all Flight Test Devices. Gently aspirate 100 male mosquitoes of the same age with a manual aspirator."
1:28-1:37 "Release them via the small hole at the bottom into the large containment tube of the Flight Test Device. Use the containment box's netted opening to ensure mosquitoes do not escape the Flight Test Device."
1:37-1:45 "Once all the mosquitoes are inside the containment tube, push the rubber base ring and netting upwards to cover the small hole through which mosquitoes were introduced by attaching the containment tube to the base plate. Repeat this procedure for at least 5 replicates per treatment that can be run in parallel."
1:48-2:14 "Confined within a small volume, the instinct of mosquitoes is to fly upwards, through one of the 40 flight tubes, and out into the large, containment tube."
2:19-2:25 "After two hours, cover the top of the containment tube with a petri dish of 9 cm of diameter and turn off all fans. At this point, the experiment is considered as complete."
2:26-2:32 "Slowly remove the containment tube through the net of the large circular opening in the containment box."
2:32-2:36 "When the tube is half-way out, be careful to hold the petri dish covering the top of the tube in place, to avoid any escapees."

2:36-2:41 “The mosquitoes remaining in the tube are considered as ‘Non-fliers’ whereas the ones remaining in the containment box are considered as ‘fliers” to calculate the flight rate.”

Mosquito compaction box (100males/$cm^3$) using 1×1×1 cm compaction boxes for adults (1-day-old)

Figure 2. Compacted males (100/$cm^3$)